PHYSIOLOGIGAL RESPONSES OF FISH TO ACID EXPOSURE

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PHYSIOLOGICAL RESPONSES OF FISH TO ACID EXPOSURE

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aan mijn ouders

aan Iris

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CHAPTER 1:

INTRODUCTION

This thesis deals with the effects of acid exposure on the physiology of carp (Cyprinus carpio) and tilapia (Oreochchromis mossambicus). In the last decades a lot of physiological and ecological research has been performed on the effects of water acidification on fish, as the dominant aquatic animals of economic importance. A broad interest in this topic was developed on account of the progressive acidification of natural waters by "acid rain".

Acid rain is a term that covers a set of highly complex environmental problems. The term is misleading since more than sixty percent of the total acid deposition is in the dry state, as gases or as small particles. Acid deposition from the atmosphere is a threat to ecosystems, especially where aquatic life is concerned, in streams and lakes in northern Europe and in some parts of North America (see Brown & Sadler, 1989; Turnpenny, 1989, for reviews).

Evidence for the gradual acidification of lakes over longer periods comes from the analysis of acid-sensitive species of diatoms from radioactively-dated lake sediments (Battarbee et al., 1985). It has been found that the water pH in a number of lochs in Scotland decreased between 0.5 and 1.2 pH units over the last 150 years. For instance the pH of a lake called Round Loch remained more or less constant at 5.5 between 1600 and 1850 but thereafter steadily declined to 4.8 in 1973, the decline being particularly rapid since 1900. The decreases in pH since the industrial revolution are likely to be due mainly to increased acidic deposition from the atmosphere (Table 1).

Table 1: Annual emissions of SO_2 or NO_x ($\cdot 10^9$ kg/yr) in the UK.

	1900	1950	1960	1970	1980	1984
SO_2	1.4	2.3	2.8	3.0	2.33	1.77
NO_x	.21	.30	.41	.50	.54	.56

Once emitted into the atmosphere the pollutants are carried and dispersed by atmospheric motions. The pollutants may travel for hundreds of kilometres during which time the gases SO_2 and NO_x are converted into products such as sulphuric and nitric acid.

Usually "acid water" is defined as water with a pH below 5.6, the pH of pure water in equilibrium with atmospheric carbon dioxide (Turnpenny, 1989). Almost worldwide, even in places as remote as the polar regions, rain and meltwater are more acidic, with mean pH values of 5.0 or below. This shows the ubiquity of acidic pollutants. Little of the water feeding streams and lakes arrives directly from the atmosphere. Most of it first flows over the land surface or through the soil. If the rain or meltwater of low pH passes for instance through sandy soil poor in base minerals (e.g. Ca/Mg carbonates and bicarbonates), or over hard granitic rocks, the rain water will be only partially neutralized by cation exchange. On the other hand, in base-rich soils, protons in the acid water are either neutralized by bicarbonate ions or removed by exchange with cations such as Mg^{2+}, K^{+}, Na^{+} and Al^{3+}.

Fish have been shown to exist in water with a wide pH range, from pH 3.5 to 11. Two types of fishery problems associated with acid water have been reported in the literature: massive fish kills associated with acid episodes, and gradual decline of fish populations in chronically acidified waters.

Instant fish kills which can be unequivocally blamed on acid episodes have seldom been reported. However, this does not imply that such phenomena seldom occur. It is just difficult to reveal the connection between the two events. The main difficulty is that pulses of acidity tend to be brief, usually following heavy rainfall or the first flush of a snowmelt (Dempsey, 1985), whilst mortalities of fish may be delayed or go unnoticed until some time after the event. A major fish kill associated with acidity was detected on the River Esk (UK) in June 1980, when a severe mortality of fresh-run adult and juvenile sea trout (Salmo trutta L.) and a salmon species (Salmo salar L.) was reported after heavy rainfall, following a dry period (Prigg, 1983). Such conditions have been shown elsewhere

to increase the acidity of runoff as a result of acid deposition (dry or via cloud, fog or mist) accumulating on catchment vegetation (Miller & Miller, 1980; Dollard, Unsworth & Harve, 1983). From other reports it is evident that changes in pH and other parameters can be quite fast, and that peak values may be sustained only for periods of hours or even minutes (Jones et al., 1983).

Field experiments have shown that the value of water pH reached is not the decisive factor in fish mortalities. In an experiment of Muniz & Leivestad (1979), aluminium-rich stream water became acutely toxic to brook trout (Salvelinus fontinalis) when the pH was raised from 4.4 to 4.9 or above. In the pH range 4.9 - 5.5 aluminium becomes very toxic to fish as a result of the formation of aluminium hydroxides (Witters, 1990). Aluminium toxicity may well play a major part in fish kills, but other metals (e.g. iron, manganese, cadmium, copper, lead, nickel, and zinc) might also contribute to the decline of fish populations (see McDonald, Reader & Dalziel, 1989).

Acid episodes probably influence the behaviour patterns of the fish concerned. There is evidence that fish show avoidance reactions to low pH conditions, and it is possible that during acid episodes, particularly in large water bodies, they can find areas of more favourable conditions, e.g. deeper water or upwelling groundwater (Gunn, 1986). In the fish kill reported by Skogheim et al. (1984), salmon spawners were seen moving downstream, the reverse of their expected behaviour pattern, away from the source of the aluminium-rich water.

Chronic water acidification by acid deposition was first mainly a problem in the poorly buffered surface waters in Scandinavia and north-east America. Field observations in these areas have shown that long-term gradual acidification of rivers and lakes causes impoverishment of fish communities and a low productivity or even the loss of entire fish assemblages (Overrein et al., 1980). In these waters salmonids are the dominant fish group. Understandably, most research on the effects of acid exposure on fish has been done on salmonids. With a continuing acid deposition, water acidification becomes

increasingly a problem in the more buffered aquatic ecosystems of Western Europe. The dominating fish species in these waters belong mainly to the group of cyprinids. The common carp (Cyprinus carpio)is a representative member of this group. For this reason, more laboratory and field research is needed on this group of fish. Field observations, involving many other fish species, have generally confirmed earlier ideas based on salmonids. For instance in a field study in Dutch waters (Leuven et al., 1987) involving 17 fish species, it was found that 8 of them occurred in water of below pH 5.0, whereas only 2 survived extremely acidified water (pH < 4.0). In the observed species, recruitment failure already tookplace at pH values above the lethal threshold. The lowest pH for occurrence and successful reproduction of carp, for instance, was found to be 4.7 and 5.4, respectively (Leuven et al., 1987).

There is a vast amount of laboratory data on the physiological effects of acid exposure on fish (see Wood, 1989, for review). The key toxic mechanism of low water pH was found to be the disturbance of electrolyte balance at the gills, and not internal acidosis. A net H^+ influx can only take place in combination with a net cation efflux in excess of a net anion efflux. So, as discussed by Wood (1989), internal acidification caused by inflow of hydrogen ions will only occur in combination with ion losses, never alone. Thus, sublethal exposure of fish to acid water often leads to hypoosmolarity of the blood, mainly caused by reduced sodium and chloride levels. Hyperglycemia, which is also often found under acid conditions, compensates to some extent for the decreased osmolarity caused by ion losses. In severe cases, ionoregulatory failure triggers circulatory collapse which finally causes death (Wood & McDonald, 1982). To compensate for the extensive ion losses, the fish decreases its plasma volume. In addition, the size and amount of red blood cells is increased by swelling and spleen contraction, respectively. Altogether, these changes cause great increases in haematocrit values, which eventually lead to cardiovascular failure. Under extreme acid conditions (pH < 4.0), fish also suffer from impaired gas exchange, indicated by increased plasma

lactate levels (Ultsch et al., 1981). Gill surfaces of fish are covered with a thick mucus layer under these circumstances. At a environmentally more realistic pH, however, fish do not become hypoxic (Ultsch et al., 1980; Wood & McDonald, 1982). Lactate accumulation, a sensitive index of oxygen delivery problems, is usually small or even absent.

Both external and internal factors influence the physiological effect of acid water on fish. Important external factors are the water calcium concentration, the presence of heavy metals, handling, and the rate of change of water pH. Water calcium is protective against ionoregulatory disturbances (McDonald, 1983), but on the other hand, blood metabolic acidosis increases with increasing water [Ca^{2+}] (Wood, 1989). The presence of heavy metals (especially aluminium), in general, deteriorates the effects of low water pH (see McDonald et al., 1989, for review). Balm (1986) found that handling stress in tilapia (Oreochromis mossambicus) in acid water impaired osmoregulation whereas handling had no effect under control conditions. Using the same species, Wendelaar Bonga et al. (1987) demonstrated that an instant drop of water pH to pH 4.0 (the rate of water acidification was 18 units per hour) can lead to substantial structural damage of the branchial epithelium, while at a lower rate of acidification (3 pH units per hour) this effect was not observed. It is clear that after gill damage ion losses can be expected to increase.

An important internal factor, which has received relatively little attention in the literature, is the endocrine response to acid exposure (see Wendelaar Bonga & Balm, 1989, for review). The endocrine system is extremely important for the control of physiological changes involved in the adjustment of animals to changes in their environment. As was discussed above, water acidification affects many aspects of fish physiology. As a consequence, pronounced and multiple responses of the endocrine system can be expected. Since a disturbed ion balance is the predominant effect of acid water on fish, it is not surprising that the hormones with osmoregulatory actions, such as cortisol, are of great importance for the endocrine response. In fish,

cortisol has been shown to regulate active ion transport mechanism in branchial and other epithelia (Dharmamba, 1979; Balm, 1986). It can also induce hyperglycemia, which contributes to the maintenance of plasma osmolarity (Mc Donalds, 1983). In rainbow trout, Oncorhynchus mykiss, low water pH caused a transient increase in plasma cortisol at pH 4.8 (Goss & Wood, 1988), but no significant change in this parameter at pH 5.0 (Witters, 1990). Combined low pH and aluminium exposure, however, caused permanent increased cortisol levels until death (Goss & Wood, 1988). After 2 days of acid exposure of tilapia, Oreochromis mossambicus, Balm (1986) found a higher release of cortisol by the headkidneys. Because plasma levels of this hormone were similar to those of the controls, he postulated that acid exposure would increase the metabolic clearance and turnover rate of cortisol.

The alarm phase of a stress response is often associated with a rise of catecholamines (see Mazeaud and Mazeaud, 1981, for review). Plasma catecholamine concentrations in fish were shown to rise in response to exhaustive exercise (Ristori and Laurent, 1985, Van Dijk & Wood, 1988), hypercapnia (Vermette & Perry, 1988), acid infusion (Boutilier et al., 1986), or hypoxia (Boutilier et al., 1988). An increase was also found with rainbow trout after exposure to pH 4.0 (Ye et al., 1991), whereas at pH 5.0 no significant change was observed (Witters, 1990). In the latter study, it was demonstrated that combined acid (pH 5.0) and aluminium exposure caused a tenfold increase in plasma adrenaline and noradrenaline. Perry et al. (1989) demonstrated that in rainbow trout, during hypercapnic acidosis, hypoxemia is the proximate stimulus for the release of catecholamines in the circulation. As outlined by Nikinmaa (1986) and Vermette and Perry (1988), the adrenergic response in fish can include at least three factors: stabilisation of red blood cell pH, an increase of blood oxygen carrying capacity, and a rise in blood pO_2.

Most laboratory studies on the effects of acid water on the physiology of fish were performed on resting animals. Fish in the wild, however, swim actively in a variety of normal

behaviours such as feeding, avoiding predators, spawning, or migrating; so studies on exercising fish are more realistic from an environmental point of view. As was demonstrated by Gonzalez & McDonald (1992), sodium losses across the gills of rainbow trout increased substantially whenever oxygen consumption increased. So the physiological disturbances of acid exposure during exercise, when oxygen uptake is higher than at rest, can be expected to be greater than those in resting fish.

Most experiments of this thesis were performed on common carp (Cyprinus carpio), which was introduced above. Part of the investigations were carried out with the cichlid tilapia (Oreochromis mossambicus), which originates from East Africa but has now been introduced in tropical and subtropical areas of most continents. It can survive heavily polluted and acidified water, which made it a successful candidate for fish farming. This quality makes it also an interesting fish species for research on acid toxicity. Another important reason why we choose carp and tilapia for our experiments is that relatively a lot is known about their physiology.

Outline of the present study

The investigations centered around physiological disturbances in common carp, Cyprinus carpio, and tilapia, Oreochromis mossambicus, after gradual water acidification in resting (chapters 2 & 3) and exercising (chapters 4, 5 & 6) states. First the possible changes in energy metabolism, of plasma pH, and of muscle, and of gill intracellular pH of tilapia after acid exposure were studied (chapter 2). In the next chapter, we investigated whether the disturbances of ion and acid/base status in carp occurring after instantaneous acidification (as reported in the literature) would also take place after a gradual acidification procedure. In chapter 4, it was tested whether acid exposure in combination with steady state exercise would enhance the impact of acid water on the physiology of resting carp. In the studies described in chapters

3 and 4, plasma cortisol and catecholamine levels were monitored during the experiments to increase our knowledge of the endocrine response to acid exposure. The effects of water acidification on the oxygen consumption pattern of both carp and tilapia are discussed in chapter 5. The final part of the investigations deals with the ability of carp to perform physical exercise after four weeks of pre-exposure to acid water (chapter 6).

REFERENCES

Balm, P.H.M. (1986). Osmoregulation in teleosts by cortisol and prolactin. Adaptation to low pH environments. Ph.D. thesis, University of Nijmegen, The Netherlands.

Battarbee, RW., Flower, R.J., Stevenson, A.C. and Rippey, B. (1985). Lake acidification in Galloway: a paleoecological test of competing hypotheses. Nature (Lond.), 314, 350-352.

Boutilier, R.G., Iwama G.K. and Randall D.J. (1986). The promotion of catecholamine release in rainbow trout, Salmo gairdneri by acute acidosis: interactions between red cell pH and haemoglobin oxygen-carrying capacity. J. Exp. Biol. 123, 145-157.

Boutilier, R.G., Dobson, G., Hoeger, V. and Randall, D.J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (Salmo gairdneri): metabolic and respiratory adaptation. Resp. Physiol. 71, 69-82.

Brown, D.J.A. and Sadler, K. (1989). Fish survival in acid waters. In Acid toxicity and aquatic animals (ed. R. Morris, D.J.A. Brown, E.W. Taylor, & J.A. Brown), pp. 31-44. Cambridge: Cambridge University Press.

Dempsey, C.H. (1985). A review of the effects on fish of changes in water chemistry during episodes of lowered pH. CERL Report TPRD/L2869/N85 Leatherhead, Surrey,UK: Central Electricity Research Laboratories.

Dharmamba, M. (1979). Corticosteroids and osmoregulation in fishes. Proc. Indian natn. Sci. Acad. B 45, 515-525.

Dollard, G.J., Unsworth, M.H. and Harve, M.J. (1983). Pollutant transfer in upland regions by occult precipitation. Nature (Lond.), 302, 241.

Gonzalez, R.J. and Mc Donald, D.G. (1992). The relationship between oxygen consumption and ion loss in a freshwater fish. J. exp. Biol. 163, 317-332.

Goss, G.G., & Wood, C.M. (1988). The effects of acid and acid aluminium exposure on circulating plasma cortisol levels and other blood parameters in the rainbow trout, Salmo gairdneri. J. Fish Biol. 32, 63-76.

Gunn, J.M. (1986). Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Envir. Biol. Fish., 17, 241-252.

Jones, H.C., Noggle, J.C., Young, R.C., Kelly J.M., Olem, H., Ruane, R.J., Pasch, R.W., Hyfantis, G.J. and Parkhurst, W.J. (1983). Investigations of the course of fishkills in fish-rearing facilities in Raven Fork watershed. TV A/ONR/WR-83/9. Tennessee Valley Authority, Division of Air and Water Resources.

Leuven, R.S.E.W., Wendelaar Bonga, S.E., Oyen, F.G.F. and Hagemejer, W. (1987). Effects of acid stress on the distribution and reproductive success of freshwater fish in Dutch soft waters. Ann. Soc. R. Zool. Belg. 117(1), 231-242.

Mazeaud, M.M. and Mazeaud, F. (1981): Adrenergic responses to stress in fish. In Stress in fish. (ed. A.D. Pickering), pp. 49-55. New York: Academic Press.

McDonald, D.G. (1983). Interaction of environmental calcium and low pH on the physiology of the rainbow trout Salmo gairdneri. I. Branchial and renal net ion and H^+ fluxes. J. Exp. Biol. 102, 123-140.

McDonald, D.G., Reader, J.P. and Dalziel, T.R.K. (1989). The combined effects of pH and trace metals on fish ionregulation. In Acid Toxicity and Aquatic animals (eds. R. Morris, D.J.A. Brown, E.W. Taylor, and J.A. Brown), pp. 221-242. Cambridge: Cambridge University Press.

Miller, H.G. and Miller, J.D. (1980). Collection and retention

of atmospheric pollutants by vegetation. In Proc. Int. Conf. Ecological Impact of Acid Precipitation. (ed. D. Drablos and A. Tollan) Oslo-As: SNSF Project.

Muniz, I.P. and Leivestad, H. (1979). Langstidseksponering av fisk til surt vann. Forsok med bekkeroye Salvelinus fontinalis Mitchill. IR 44/79. Oslo-As: SNSF.

Nikinmaa, M. (1986). Control of red cell pH in teleost fishes. Ann. Zool. Fenn 23, 223-235.

Overrein, L.N. (1980). Acid precipitation - impact on the natural environment. Proc. 11th World Energy Conf. Munich. 570-583.

Perry, S.F., Kinkead, R., Gallaugher, P., & Randall, D.J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (Salmo gairdneri). Respiration Physiology 77, 351-364.

Prigg, R.F. (1983). Juvenile salmonid populations and biological quality of upland streams in Cumbria, with particular reference to low pH effects. North West Water, Report no. BN 77-2-83. Carlisle, UK: North West Water, Rivers Division.

Ristori, M.T. and Laurent, P. (1985). Plasma catecholamines and glucose during moderate exercise in the trout: comparison with bursts of violent activity. Exp. Biol. 136, 247-253.

Skogheim, O.K., Rosseland, B.O. and Sevaldrud, I. (1984). Deaths of spawners of Atlantic salmon in River Ogna, S.W. Norway, caused by acidified aluminium-rich water. Rep. Inst. Freshwater Res. Drottningholm, 61, 195-202.

Turnpenny, A.W.H. (1989). Field studies on fisheries in acid waters in the UK. In Acid Toxicity and Aquatic animals (ed. R. Morris, D.J.A. Brown, E.W. Taylor, & J.A. Brown), pp. 221-242. Cambridge: Cambridge University Press.

Ultsch, R.G., Ott, M.E. and Heisler, N. (1980). Standard metabolic rate, critical oxygen tension, and aerobic scope for spontaneous activity of trout (Salmo gairdneri) and carp (Cyprinus carpio) in acidified water. Comp. Biochem. Physiol. 67, 329-335.

Ultsch, R.G., Ott, M.E. and Heisler, N. (1981). Acid-base and electrolyte status in carp (Cyprinus carpio) exposed to low environmental pH. J. exp. Biol. 93, 65-80.

Van Dijk, P.L.M. and Wood, C.M. (1988). The effect of adrenergic blockade on the recovery process after strenuous exercise in the rainbow trout, Salmo gairdneri, Richardson. J. Fish Biol. 32, 557-570.

Vermette, M.G. and Perry, S.F. (1988). Adrenergic involvement in blood oxygen transport and acid-base balance during acidosis in the rainbow trout, Salmo gairdneri. J. Comp. Physiol. 158, 107-115.

Wendelaar Bonga, S.E., Flik, G. and Balm, P.H.M. (1987). Physiological adaptation to acid stress in fish. Ann. Soc. R. Zool. Belg.117, 243-254.

Wendelaar, S.E. and Balm, P.H.M. (1989). Endocrine responses to acid stress in fish. In Acid toxicity and aquatic animals (eds. R. Morris, D.J.A. Brown, E.W. Taylor, and J.A. Brown) pp. 243-263. Cambridge: Cambridge University press.

Witters, H. (1990). Physiological study on the waterbug, Corixa punctata and the trout Oncorhynchus mykiss: affects of aluminium in acid water. Ph.D. thesis, University of Antwerp, Belgium.

Wood, C.M. (1989). The physiological problems of fish in acid waters. In Acid Toxicity and Aquatic animals (eds. R. Morris, D.J.A. Brown, E.W. Taylor, and J.A. Brown), pp. 125-152. Cambridge: Cambridge University Press.

Ye, X., Randall, D.J., & He, X. (1991). The effect of acid water on oxygen consumption, circulating catecholamines and blood ionic and acid-base status in rainbow trout (Salmo gairdneri, Richardson). Fish Physiol. Biochem. 9, 15-21.

CHAPTER 2:

^{31}P-NMR STUDIES ON ACID-BASE BALANCE AND ENERGY METABOLISM OF ACID EXPOSED FISH

ABSTRACT

Tilapias (*Oreochromis mossambicus*), acclimated to 25 °C and water with a Ca^{2+} content of 0.68 mmol l^{-1}, were subjected to gradual water acidification (from pH 7.6 to 4.0 in 4 h), followed by 10 h of exposure to low pH (pH 4.0), gradual environmental alkalization (from pH 4.0 to 7.6 in 2 h) and 6 h of recovery at normal pH (pH 7.6). Intermediates of energy metabolism were measured in perchloric acid extracts of gill, muscle and blood. In a separate series of experiments, the intracellular pH (pHi) and the levels of high-energy phosphate compounds were continuously monitored by *in vivo* ^{31}P-NMR spectroscopy. We used a 10 mm surface coil, which was positioned above the gill arches or the epaxial white muscle. With the coil above the gill, splitting of the inorganic phosphate peak indicated that the signal was picked up from three different compartments. These were tentatively identified as plasma, muscle and gill epithelium. Water acidification induced a transient pH drop of the plasma (0.24 units) and the gill (0.19 units) but the pH of both compartments slowly recovered during the 10-h exposure to acid water. In contrast, the pHi of muscle tissue was only slightly affected. Alkalization of the environment caused a surprising transient decline of the plasma pH, which was not due to lactic acidosis, but may be related to the precipitation of a buffer compound like $CaCO_3$ in scales and bone. The high-energy phosphate stores in the tissues were unchanged during the whole experimental protocol.

Introduction

The disappearance of many fish species from strongly acid waters (pH<5) has aroused much interest in the physiological effects of acid exposure. Most studies have focused on electrolyte (Na^+, Cl^-) losses and acidaemia due to increased

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permeability of the gill membrane In contrast, reports on disturbances of energy metabolism have been relatively scarce Lowering of the environmental pH is known to elevate blood glucose levels (Audet *et al* 1988, Audet and Wood, 1988, Brown *et al* 1984, Lee *et al* 1983, Tam *et al* 1988), plasma amino acid levels (Fugelli and Vislie, 1982, Tam *et al* 1988) and protein levels (Audet *et al* 1988, Brown *et al* 1984, Tam *et al* 1988) Blood lactate levels are sometimes increased (Höbe *et al* 1984, Ultsch *et al* 1981), but are usually unaffected (Neville, 1979, Ultsch *et al* 1981) Ammonia excretion is enhanced (Audet and Wood, 1988, King and Goldstein, 1983, Ultsch *et al* 1981), cortisol turnover increases (Balm, 1986), amino acid catabolism is activated (Balm, 1986) and more glycogen is stored in the liver (Lee *et al* 1983, Murthy *et al* 1981*b*) This pattern of changes suggests an increase in the importance of protein as a metabolic fuel and an increase in gluconeogenesis (Murthy *et al* 1981*a*,*b*, Tam *et al* 1988) Environmental acidification can also impair oxidative metabolism and reduce high-energy phosphate content A decline in ATP levels and a decreased adenylate energy charge have been observed in the tissues of the gulf killifish (*Fundulus grandis*), with the gill epithelium showing the most prominent loss of nucleoside triphosphate (MacFarlane, 1981)

The toxicity of environmental acid is dependent on additional factors Water hardness reduces H_2SO_4 toxicity (Graham and Wood, 1981, McDonald, 1983, McDonald *et al* 1980, 1983) The physiological effects are more severe in fish transferred directly to low-pH water than in animals gradually exposed to acid (Stuart and Morris, 1985, Wendelaar Bonga *et al* 1987)

The present paper seeks to evaluate the short-term effects of acid stress on freshwater fish using *in vivo* ^{31}P-NMR spectroscopy and enzymatic methods The animals were acclimated to water of relatively low calcium content (0 68 mmol l^{-1}) and gradually exposed to acid to simulate natural environmental conditions Transient declines of pHi were observed upon acid exposure without any major disturbance of energy metabolism

Materials and methods

Animals

Laboratory-reared tilapias (*Oreochromis mossambicus*, 80±15 g body mass, length 14±2 cm) were used in all experiments They were acclimated to 25 °C, a 16 h light period, normal oxygen levels (P_O, 17–21 kPa) and 'diluted tap water' (1 vol of copper-free tap water 3 vols of demineralized water, pH 7 6–7 8, Ca^{2+} 0 68, Mg^{2+} 0 16, Na^+ 0 83, K^+ 0 07, NO_3^- 0 03, HCO_3^- 1 11, SO_4^{2-} 0 29, SiO_2 0 06, Cl^- 0 92 mmol l^{-1}) The experimental animals were fed daily with cichlid food in flake form (Lapis, Europet, Nurnberg, FRG) The anaesthetic ethyl-*m*-aminobenzoate methanesulphonate (MS 222) and Tris buffer were products of Sigma (St Louis, MO)

Conditioning

At the onset of this study, the experimental fish were divided into two groups

Group 1 was placed directly into a holding tank with conditions as described above. These animals were used for *in vivo* ^{31}P-NMR studies of the epaxial muscle, and for metabolite measurements. The caudal part of the operculum of the animals in group 2 was surgically removed under MS 222 anaesthesia. After surgery, the fish were treated with a solution of potassium dichromate (1 25000 w/v, for 10 days) as a preventive measure against infections. Finally, they were put in a separate holding tank and treated in the same way as group 1. Fish from group 2 were used for *in vivo* ^{31}P-NMR studies of the gill.

In vivo *^{31}P-NMR studies*

For each experiment, a single fish was kept overnight in a darkened tank. On the day of the experiment, the fish was anaesthetized by addition of Tris–MS 222 (adjusted to the pH of the environment) to the water to a final concentration of 120 p.p.m. As soon as the fish lost equilibrium (<10 min), it was mounted in the Perspex flow cell of the *in vivo* ^{31}P-NMR probe (Van den Thillart *et al.* 1989*a*) which was connected to equipment for the control of temperature, pH and P_{CO_2} (Fig. 1). During the measurements the animal was immobilized by an inflatable plastic bag filled with water. Water temperature was maintained at 25 °C, using a heater with digital temperature readout and the gills were continuously irrigated with diluted tap water (see above). Since the water did not contain MS 222, the fish recovered rapidly (<2 min) and remained conscious during the experiment. The environmental pH was controlled with a pH-stat and a digital pH-meter (Metrohm, Herisau, Switzerland). The pH-stat used either 0.5 mol l^{-1} H_2SO_4 or 1 mol l^{-1} NaOH.

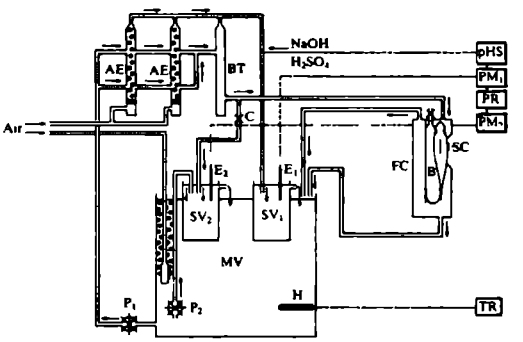


Fig. 1. Scheme of the experimental apparatus used in the *in vivo* ^{31}P-NMR experiments. AE, aerators, B, inflatable plastic bag, BT, bubble trap, C, clamp, E_1 and E_2, pH electrodes, FC, flow cell, H, heater, MV, main mixing vessel, P_1 and P_2, pumps, pHS, pH-stat, PM_1 and PM_2, pH-meters for measurement of the pH in the mixing vessel and the pH of the gill perfusion fluid, respectively, PR, penrecorder, SC, surface coil, SV_1 and SV_2, secondary mixing vessels, TR, digital temperature readout.

The water flowing through the NMR probe was thoroughly aerated to avoid hypercapnia during titration with acid. We used two special aerators and a third aerator in the main mixing vessel. Preliminary experiments showed that our aeration system was effective. There was only a transient and minor rise of the P_{CO_2} (from 31 to 53 Pa) during water acidification. The water current could be adjusted with a clamp.

In vivo ^{31}P-NMR spectra of the epaxial muscle (or gill) were acquired with a Bruker MSL-400 spectrometer. The signal of the tissue of interest was picked up with a surface coil of 10 mm diameter, which was double-tuned to the hydrogen (400 MHz) and phosphorus (162 MHz) frequencies. A microsphere, filled with a solution of methylene diphosphonate in deuterium oxide, was mounted at the centre of the coil and served as an external intensity standard. The homogeneity of the stationary magnetic field was optimized by shimming on the ^{1}H-NMR signal of the intracellular water. ^{31}P-NMR spectra (8192 data points) were accumulated over a period of 30 min and consisted of 412 individual scans, using a pulse width of 60° (in the sensitive volume), an acquisition time of 0.4 s and a 4 s relaxation delay. Measurements of the longitudinal relaxation time (T_1) of phosphocreatine and ATP by the progressive saturation method indicated that the resonances of these compounds were fully relaxed [$\exp(-t/T_1)<0.1$]. Tissue pH values were calculated from the difference in chemical shift between the inorganic phosphate (P_i) and phosphocreatine resonances in the NMR spectra. The pH measurements were standardized by titration of two different model solutions over a pH range of 5.0–8.5. The model solutions were (1) inorganic phosphate and phosphocreatine dissolved in physiological saline; and (2) a homogenate of muscle tissue, as described previously (Van den Thillart *et al.* 1989*b*).

In control fish (more than 2 h elapsed after handling, exposed to well-oxygenated water of pH 7.6), the intracellular pH of the epaxial muscle was about 7.3 and the phosphocreatine/inorganic phosphate ratio was high (>15). If the pH of the water was not altered, all NMR-observable parameters remained stable for periods greater than 36 h.

The water was not acidified until a period of more than 3 h had elapsed after handling. During the final 90 min of this period, we measured control parameters for the animal. At least three subsequent 30-min spectra at the neutral steady state were accumulated before the set-point of the pH-stat was changed. The pH was gradually lowered from 7.6 to 4.0 over a period of 4 h and maintained at 4.0 for the following 10 h. The pH was then gradually raised from 4.0 to 7.6 over a period of 2 h and maintained at 7.6 for another 6 h. Spectra of the epaxial muscle (or gill) were acquired continuously during the whole sequence of events.

Metabolite measurements

In a parallel series of experiments, fish were subjected to the same protocol of water acidification, exposure to acid water and recovery from acid stress. At various times, fish were anaesthetized with Tris–MS 222, as described above, and a blood sample (0.5–1.0 ml) was drawn by cardiac puncture. About 2 g of epaxial

white muscle was excised from each fish (below the dorsal fin) and immediately freeze-clamped The gills were cut out and dropped into liquid nitrogen The removal and freezing of the tissues was completed within 30 s Gill arches were discarded after separation from the lamellae at the temperature of liquid nitrogen Blood samples, gill lamellae and muscle pieces were extracted with ethanol/ perchloric acid, as described previously (Van den Thillart *et al* 1982) Creatine, phosphocreatine, ATP, glucose and lactate were measured in the tissue extracts (Bergmeyer, 1970, Van den Thillart *et al* 1982).

Statistics

Differences between groups were tested using the nonparametric test of Wilcoxon A dual-tail probability of <0 05 was considered statistically significant

Results

Appearance of NMR spectra

When the surface coil was placed above the epaxial muscle, we acquired *in vivo* ^{31}P-NMR spectra of excellent resolution and signal-to-noise ratio These results have been published previously (Van den Thillart *et al* 1989*a*, Van Waarde *et al* 1990) and are not reproduced here A representative spectrum obtained with the coil above the gill is presented in Fig 2 The most striking feature of the spectrum is the splitting of the inorganic phosphate resonance, indicating that the signal is picked up from three different compartments

Changes of the pHi induced by environmental acidification

The protocol of gradual water acidification, exposure to low pH, gradual environmental alkalization and recovery at pH 7 6 induced disturbances in the pHi of the experimental fish The pH of each compartment could be calculated from the difference between the chemical shift of its inorganic phosphate resonance and the phosphocreatine resonance in the NMR spectra (Fig 2, Van den Thillart *et al* 1989*b*) The observed pattern is illustrated in Fig 3 and a statistical treatment of the data is presented in Table 1

The pH of all compartments was lowered at the end of the 4-h period of environmental acidification The pH drop was largest in compartment 1 (from 7 78 to 7 54), intermediate in compartment 3 (from 6 98 to 6 79) and small in compartment 2 (from 7 27 to 7 21) During prolonged exposure to low-pH water, the pH of all compartments returned towards the control value After 10 h at pH 4, this return was complete in compartments 1 and 2, but only partial in compartment 3 Subsequent alkalization of the environment induced a new fall of the pH of compartment 1, whereas the pH of compartment 2 was unaffected and that of compartment 3 returned towards the control value During 6 h of recovery in neutral water, all NMR-observed pH values returned to normal

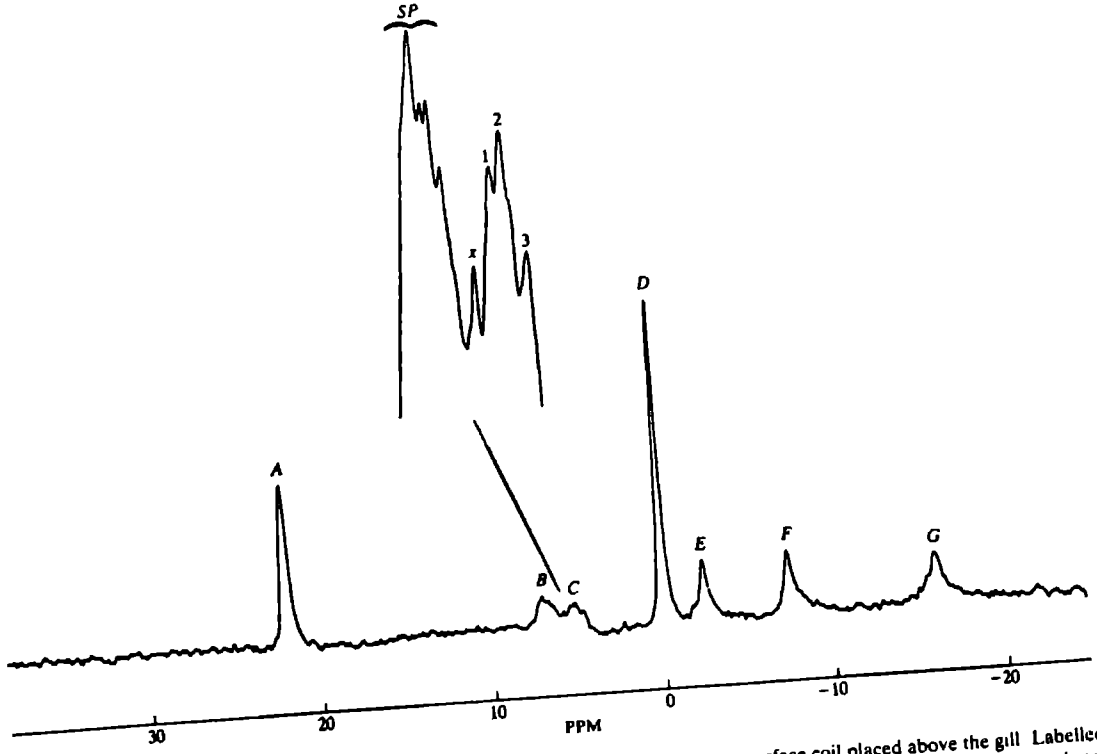


Fig 2. *In vivo* ^{31}P-NMR spectrum of *Oreochromis mossambicus*, acquired with a 10 mm surface coil placed above the gill. Labelled resonances are those of: *A*, methylene diphosphonate (external standard); *B* sugar phosphates; *C* inorganic phosphate; *D* phosphocreatine; *E,F,G* gamma-, alpha- and beta-phosphate atoms of ATP. The inset shows the splitting of the inorganic phosphate peak. *SP*, sugar phosphates; *x*, unknown resonance, 1,2,3, inorganic phosphate in three different compartments with different pH values. PPM means parts per million (1 p.p.m. is 162 Hz).

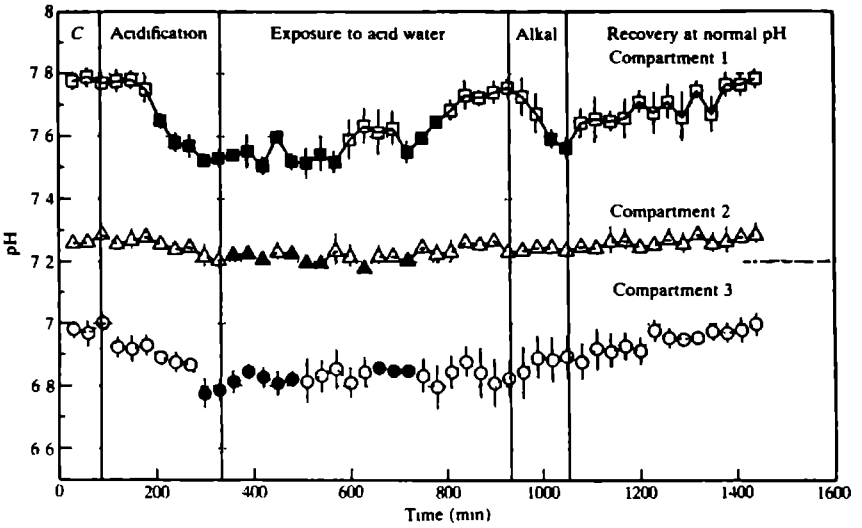


Fig 3 pH changes in three compartments of *Oreochromis mossambicus* caused by changes in the acidity of the environment The pH of the compartments was measured by *in vivo* ^{31}P NMR spectroscopy using a surface coil above the gill Each data point is the mean of four independent observations on four different animals Significant differences between an experimental value and the value obtained before acidification (Wilcoxon s Q test $P<0.05$) are indicated by filled symbols The vertical lines indicate standard errors some values acquired between 500 and 900 min are not significantly different from the control owing to large individual variability C, control Alkal, alkalization

Changes in direct energy reserves, glucose and lactate induced by acid water

During the *in vivo* ^{31}P NMR experiments on gill and muscle tissue, we never observed a significant depletion of tissue ATP and phosphocreatine Since the origin of the phosphocreatine and ATP signals in the ^{31}P-NMR spectra is not well defined, we also measured intermediates of energy metabolism in perchloric acid extracts of muscle (Table 2), gill (Table 3) and blood (Table 4)

Acid exposure had no influence on the phosphocreatine, creatine and ATP contents of white muscle, but there was a tendency towards lower lactate levels in the acid-treated group (Table 2) After 2 h in pH 4 water, and at the end of the 2-h period of alkalization, this trend was statistically significant The lower lactate level is probably due to a suppression of routine activity We always observed that the fish in the acid tank became quiet and swam slowly, in contrast to the control animals which showed large peaks of activity

A dramatic decline of muscle phosphocreatine and ATP occurred in both the control and acid exposed fish during the 6 h recovery period (Table 2) The reason

Table 1 *Influence of water acidification on intra- and extracellular pH of tilapia*

Condition	Surface coil above the gill			Surface coil above the epaxial muscle
	Compartment 1	Compartment 2	Compartment 3	Single compartment
Control (pH 7 6)	7 78±0 06 (12)	7 27±0 03 (12)	6 98±0 07 (12)	7 30±0 05 (8)
End of acid titration	7 54±0 07* (12)	7 21±0 05† (12)	6 79±0 08* (12)	7 21±0 04† (8)
10 h of acid exposure (pH 4)	7 74±0 07 (12)	7 25±0 04 (12)	6 90±0 03† (12)	7 31±0 05 (8)
End of base titration	7 56±0 07* (8)	7 24±0 04 (8)	6 95±0 08 (8)	7 29±0 06 (8)
6 h of recovery (pH 7 6)	7 76±0 08 (12)	7 27±0 06 (12)	6 97±0 07 (12)	7 30±0 06 (8)

All pH values were determined by *in vivo* ^{31}P-NMR spectroscopy

Compartments 1, 2 and 3 are tentatively identified as blood plasma, muscle and gill epithelium (see Discussion)

* † Significant difference between the experimental pH value and the control value before acidification (* $P<0.001$, † $P<0.01$, Wilcoxon's Q-test, dual-tail probability) Values are mean±S D

Values in brackets refer to the number of observations on four different animals

10 h of acid exposure and 6 h of recovery mean the end (i e the final hour) of these periods, not the average value for the entire period

Table 2. *Influence of water acidification on energy metabolism of white muscle*

Condition	Phosphocreatine		Creatine		ATP		Lactate	
	Control	Acid	Control	Acid	Control	Acid	Control	Acid
Control (pH 7 6)	11 82±1 13	12 31±1 65	14 80±2 07	16 54±0 52	4 10±0 59	4 13±0 24	6 28±0 49	6 73±1 74
End acid titration	10 06±4 00	8 26±0 76	19 34±2 22	18 93±1 14	3 33±0 73	3 11±0 95	7 52±2 20	5 73±0 86
2 h of acid exposure	10 96±3 61	12 87±2 16	21 07±2 92	16 28±1 10	3 38±1 07	3 82±1 00	10 11±1 09	5 96±1 34*
10 h of acid exposure	13 33±0 15	10 88±1 55	12 58±1 43	11 27±3 86	3 89±0 24	3 29±1 16	5 11±1 15	3 93±1 97
After base titration	11 86±3 38	14 03±0 92	15 15±1 63	14 20±4 20	3 87±0 27	3 88±0 35	8 91±3 00	2 99±0 75*
6 h of recovery	4 70±1 05	5 95±3 28	19 41±2 52	18 86±4 22	2 33±0 45	2 72±1 00	9 55±1 61	7 77±1 65

The acid group was exposed to the sequence of experimental conditions listed on the left, the control group was kept continuously in pH 7 6 water

Control animals were killed simultaneously with acid exposed individuals to compensate for diurnal effects Values are mean±S D of four observations on four different animals, concentrations are expressed as μmol g^{-1} wet mass

* Significant difference between control and acid-exposed animals 2 h or 10 h of acid exposure actually means 4 h of acid titration followed by 2 h or 10 h of exposure to pH 4 water

Table 3 *Influence of water acidification on energy metabolism of gill tissue*

Condition	Phosphocreatine	Creatine	Lactate
Control (pH 7 6)	0 71±0 17 (8)	0 71±0 27 (8)	0 55±0 32 (8)
10 h of acid exposure (pH 4)	0 51±0 18 (3)	0 70±0 31 (3)	0 45±0 14 (3)
After base titration	0 53±0 17 (3)	0 75±0 22 (3)	0 38±0 17 (3)

Values are mean±S D

Numbers in brackets indicate the number of independent observations on different fish Concentrations are expressed as μmol g^{-1} wet mass

No significant differences between the control and experimental groups were detected

10 h of acid exposure actually means 4 h of acid titration followed by 10 h of exposure to pH 4 water

Table 4. *Influence of water acidification on blood glucose and lactate*

	Glucose		Lactate	
Condition	Control	Acid	Control	Acid
Control (pH 7 6)	3 09±0 57	3 41±0 41	0 86±0 39	0 72±0 19
End of acid titration	4 73±0 45	4 36±1 47	0 66±0 35	0 97±0 33
2 h of acid exposure	3 93±0 42	5 98±2 22	0 63±0 18	0 40±0 04
10 h of acid exposure	3 64±0 13	4 44±1 01	0 40±0 18	0 49±0 17
End of base titration	5 38±0 58	3 64±0 93*	1 01±0 42	0 45±0 11*
6 h of recovery (pH 7 6)	5 75±1 65	3 93±0 68	1 88±0 60	1 08±0 54

The acid group was exposed to the sequence of experimental conditions listed on the left, the control group was kept continuously in pH 7 6 water

Control animals were killed simultaneously with acid-exposed individuals to compensate for diurnal effects

Metabolites were determined in neutralized perchloric acid extracts of whole blood, concentrations are expressed as μmol g^{-1} wet mass Values are mean±S D of four observations on four different animals

* Significant difference between control and acid-exposed individuals ($P<0$ 05, Wilcoxon's Q-test)

2 h or 10 h of acid exposure actually means 4 h of acid titration followed by 2 h or 10 h of exposure to pH 4 water

for this change is not clear The decrease of high-energy phosphate content coincided with the large activity peak in the morning after the lights in the aquarium room had been switched on However, hydrolysis of phosphocreatine is unlikely, since there was no accompanying increase of creatine content

Surprisingly, lactate, phosphocreatine and creatine levels in the gill lamellae were not affected by the experimental protocol (Table 3). Blood glucose and lactate levels also showed only minor changes. There was a significant difference between control and acid-exposed individuals at the end of alkalization, fish recovering from acid stress having lower levels of glucose and lactate (Table 4)

Discussion

Identification of the compartments observed by NMR

When the surface coil of the *in vivo* ^{31}P-NMR probe was above the gill, we observed splitting of the inorganic phosphate peak in the NMR spectra, indicating that the signal was picked up from three different compartments (Fig 1)

The first compartment had a control pH of 7 78±0 06 (Table 1) The following evidence suggests that it represents plasma (i) The value of 7 78 is a normal pH for blood plasma of relaxed fish at 25°C Cameron and Kormanik (1982) measured values of 7 93 at 15°C, 7 83 at 22°C and 7 70 at 31°C (ii) We performed a parallel experiment in which two cannulated tilapias were gradually exposed to environmental acid, using the same protocol of acidification Although we could not sample very frequently, we measured a similar transient pH drop (from 7 80 to 7 55) and recovery of the arterial pH during prolonged exposure to pH 4, as was observed in compartment 1 of the present study (iii) It may be argued that the alkaline inorganic phosphate peak in the NMR spectra represents mitochondrial phosphate, since mitochondria functioning normally have a lower internal H^+ concentration than the surrounding cytoplasm It is generally believed that mitochondrial phosphates are NMR-invisible (Murphy *et al* 1988), but in some studies, using isolated mitochondria (Ogawa *et al* 1978, Ogawa and Lee, 1984), hepatocytes (Cohen *et al* 1978) and perfused rat liver (Thoma and Ugurbil, 1988), distinct signals from cytoplasmic and mitochondrial phosphates have been observed The intramitochondrial pH was reported as 7 86 at 8°C (Ogawa and Lee, 1984) Nevertheless, we do not consider it likely that the first P_i resonance represents mitochondrial phosphate In ^{31}P-NMR spectra of the lateral red muscle of fish, no alkaline P_i resonance was visible, although the tissue contains many mitochondria (Van den Thillart *et al* 1989*b*) Resonances of intramitochondrial phosphate are usually 2 5–3 times broader than those of cytosolic phosphate owing to interaction with divalent metal ions (Ogawa *et al* 1978, Ogawa and Lee, 1984), but the inorganic phosphate peak of compartment 1 was relatively narrow (Fig 2) (iv) We measured inorganic phosphate levels in tilapia plasma and found a value of 1 2±0 1 mmol l^{-1} Such a concentration would indeed give rise to a small peak in the ^{31}P-NMR spectrum

The inorganic phosphate resonance of the second compartment indicated a control pH of 7 27±0 03 (Table 1) We think this compartment is muscle (with a contribution from erythrocytes) for the following reasons (i) The pH_i in myotomal muscle of relaxed tilapias is 7 27–7 30 at 25°C (Van den Thillart *et al* 1989*a*) (ii) In a parallel series of experiments using the same protocol, we placed the surface coil above the epaxial muscle and monitored changes in pH_i during acid exposure and recovery from acid stress We found a similar, transient and minor pH drop (from 7 30 to 7 21) during acidification followed by stability of the intracellular pH (Table 1) (iii) Since the intraerythrocyte pH of teleost fish is 7 22 at 25°C (Cameron and Kormanik, 1982, see also below) and the signal from plasma is being picked up, it is likely that red blood cells contribute to the P_i resonance of compartment 2

The P_i resonance of the third compartment indicated an initial pH value of 6.98±0.07 (Table 1). We have tentatively identified this compartment as gill epithelium, since other possible sources of the NMR signal could be excluded. Compartment 3 cannot be the epithelium or bone of the gill cover, since this tissue was found to have a relatively high pHi of 7.4 (these measurements were performed with an oval surface coil of 20 mm×5 mm; accumulation times of several hours were necessary owing to a low signal-to-noise ratio). Skin is not the source of the acid-shifted P_i resonance either, since this P_i resonance was not observed when the surface coil was above the myotomal muscle. Red blood cells are not causing the P_i resonance of compartment 3. In freshly drawn blood from an anaesthetized fish, we measured an intracellular pH of 7.29 and a plasma pH (pHe) of 7.57 by ^{31}P-NMR (the low pHe is probably due to the lactic acidosis induced by anaesthesia). After lysis of the cells by the freeze–thaw method, the directly measured pH values were 7.23 and 7.52, respectively. The hypothesis that compartment 3 represents the environmental water is unlikely, since the water did not contain inorganic phosphate and the environmental pH was changed from 7.6 to 4.0, rather than 7.0 to 6.8.

The mechanism underlying the transient drops of pHe and pHi during environmental acidification

During titration of the surrounding water with acid, we observed a transient fall of the pH in all three compartments (Fig. 3). In theory, three mechanisms may have caused the decline of the pH in the animal: (i) accumulation of lactic acid in the tissues due to hypoxic stress or an escape response; (ii) an increase of the CO_2 tension in the fish body due to a suppression of breathing or hypercapnia; (iii) influx of protons from the surrounding water across the gill epithelium.

We measured the lactate concentration in muscle (Table 2), gill (Table 3) and blood (Table 4) to find out whether the pH drops were due to lactic acidosis. No significant increase of the lactic acid level occurred. The transient declines of pH are therefore not a side effect of an escape reaction or caused by hypoxic stress. The second explanation for the observed acidosis does not seem very probable either. It is true that free-swimming tilapias respond to acid water with a decreased amplitude of ventilation, but the animals in this study were artificially ventilated, resulting in very efficient gas exchange. Also, there was no macroscopically visible accumulation of mucus on the gills and the P_{CO_2} of the water showed only a minor rise during titration with acid (see Materials and methods).

It can therefore be safely assumed that the transient acidosis is due to H^+ influx from the surrounding water. McWilliams and Potts (1978) observed a shift from negative to positive charge over the gill epithelial membrane upon exposure of trout to acid water, which suggests an influx of H^+. Other authors have measured a net influx of protons in acid-exposed fish (Hōbe *et al.* 1984; Holeton *et al.* 1983; McDonald and Wood, 1981; McDonald, 1983).

The acidosis that we observed in tilapias was transient (Table 1, Fig. 3). It is thus necessary to explain why the decline in pH came to an end and was actually

reversed in compartments 1 and 2 during acid exposure Two possible mechanisms may be involved (i) increased sodium pumping in the gills and kidneys (since Na^+ uptake is coupled to H^+ extrusion) and (ii) a decrease of the ion permeability of the gill membrane, causing a suppression of proton influx Both phenomena are known to occur Rainbow trout showed a marked increase in renal acid excretion during 1–4 days of exposure to pH 4 (McDonald and Wood, 1981) Balm (1986) measured an increase of the Na^+/K^+-ATPase activity in gill tissue of tilapias during a 5-day exposure to pH 3 3, but at pH 4 5 this response did not occur (Wendelaar Bonga *et al* 1990) During 1–4 days of exposure of rainbow trout to pH 4, McDonald and co-workers demonstrated a substantial decrease of net Na^+ losses and H^+ uptake, which suggests alterations in the ion permeability of the gill membrane (McDonald, 1983, McDonald *et al* 1983) Dramatic increases of ion pumping cause partial depletion of ATP and phosphocreatine and lowering of the adenylate energy charge in the gill epithelium (MacFarlane, 1981) The fact that we did not observe such changes in acid-exposed tilapias (Table 3) may therefore indicate that permeability changes were more important than changes in the activity of Na^+/H^+- and Na^+/NH_4^+-ATPases under the conditions of this study

The mechanism underlying the transient decline of pHe during environmental alkalization

Our most intriguing observation is the transient decline of the pH of compartment 1 (i e plasma) during titration of the water with base (Fig 3) This phenomenon does not occur in the other compartments In a study on the recovery of cannulated rainbow trout from acid stress (Holeton *et al* 1983), no transient decline of the blood pH was observed However, these authors did not sample earlier than 4–6 h after the beginning of base titration, so an initial decline of the blood pH may have been overlooked

It appears that protons that have entered the fish during the initial 4 h of acid exposure are temporarily stored in a buffer When the environmental pH starts to rise, stored acid equivalents are released into the blood and finally excreted It is possible that calcium carbonate from the skeleton participates in pH buffering (Holeton *et al* 1983, McDonald and Wood, 1981, Wendelaar Bonga and Dederen, 1986, Wendelaar Bonga *et al* 1984) Resynthesis of calcium carbonate upon the return to a neutral environment will be accompanied by the formation of acid equivalents It is conceivable that such repletion of a depleted buffer compound is the cause of the transient decline of the plasma pH

In conclusion, this paper shows that *in vivo* ^{31}P-NMR spectroscopy can be used to monitor the responses of pHi and pHe in fish to pH changes of the environmental water The use of the NMR technique has provided interesting new information on disturbances of the internal milieu during water acidification and recovery from acid stress First, tilapias were able to restore the pH of muscle and blood to normal values within a few hours of the onset of acid exposure Second, gradual exposure to acid did not give rise to impairment of energy metabolism in muscle and gill Finally, there was a transient decline of the plasma pH during the

gradual return to a neutral environment, which may be related to resynthesis of a buffer compound

References

AUDET, C MUNGER, R S AND WOOD, C M (1988) Long-term sublethal acid exposure in rainbow trout (*Salmo gairdneri*) in soft water effects on ion exchanges and blood chemistry *Can J Fish aquat Sci* **45**, 1387–1398

AUDET C AND WOOD, C M (1988) Do rainbow trout (*Salmo gairdneri*) acclimate to low pH? *Can J Fish aquat Sci* **45** 1399–1405

BALM, P H M (1986) Osmoregulation in teleosts by cortisol and prolactin adaptation to low pH environments DSc thesis, University of Nijmegen, The Netherlands

BERGMEYER, H U (1970) *Methoden der enzymatischen Analyse* (two volumes) Weinheim, FRG Verlag Chemie

BROWN, S B , EALES, J G , EVANS, R E AND HARA, T J (1984) Interrenal, thyroidal, and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification *Can J Fish aquat Sci* **41**, 36–45

CAMERON, J N AND KORMANIK, G A (1982) Intra- and extracellular acid–base status as a function of temperature in the freshwater channel catfish, *Ictalurus punctatus* *J exp Biol* **99** 127–142

COHEN, S M , OGAWA, S , ROTTENBERG, H , GLYNN P YAMANE T , BROWN, T R SHULMAN R G AND WILLIAMSON J R (1978) ^{31}P nuclear magnetic resonance studies of isolated rat liver cells *Nature* **273** 554–556

FUGELLI, K AND VISLIE T (1982) Physiological response to acid water in brown trout (*Salmo trutta* L) cell volume regulation in heart ventricle tissue *J exp Biol* **101**, 71–82

GRAHAM, M S AND WOOD C M (1981) Toxicity of environmental acid to the rainbow trout interactions of water hardness acid type and exercise *Can J Zool* **59** 1518–1526

HÖBE, H , WOOD, C M AND MCMAHON, B R (1984) Mechanisms of acid–base and ionoregulation in white suckers (*Catostomus commersoni*) in natural soft water I Acute exposure to low ambient pH *J comp Physiol* **154**, 35–46

HOLETON, G F , BOOTH, J H AND JANSZ, G F (1983) Acid–base balance and Na^+ regulation in rainbow trout during exposure to, and recovery from, low environmental pH *J exp Zool* **228**, 21–32

KING, P A AND GOLDSTEIN, L (1983) Renal ammonia excretion and production in goldfish, *Carassius auratus*, at low environmental pH *Am J Physiol* **245**, R590–R599

LEE, R M , GERKING, S D AND JEZIERSKA, B (1983) Electrolyte balance and energy mobilization in acid-stressed rainbow trout, *Salmo gairdneri*, and their relation to reproductive success *Envl Biol Fish* **8**, 115–123

MACFARLANE, R B (1981) Alterations in adenine nucleotide metabolism in the gulf killifish (*Fundulus grandis*) induced by low pH water *Comp Biochem Physiol* **68B**, 193–202

MCDONALD, D G (1983) The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri* I Branchial and renal net ion and H^+ fluxes *J exp Biol* **102**, 123–140

MCDONALD, D G , HÖBE, H AND WOOD, C M (1980) The influence of calcium on the physiological responses of the rainbow trout, *Salmo gairdneri*, to low environmental pH *J exp Biol* **88**, 109–131

MCDONALD D G , WALKER, R L AND WILKES, P R H (1983) The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri* II Branchial ionoregulatory mechanisms *J exp Biol* **102**, 141–155

MCDONALD, D G AND WOOD, C M (1981) Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH *J exp Biol* **93**, 101–118

MCWILLIAMS, P G AND POTTS, W T (1978) The effects of pH and calcium concentration on gill potentials in the brown trout, *Salmo trutta* *J comp Physiol* **126**, 277–286

MURPHY, E , GABEL, S A , FUNK, A AND LONDON, R E (1988) NMR observability of ATP Preferential depletion of cytosolic ATP during ischemia in perfused rat liver *Biochemistry, N Y* **27** 526–528

MURTHY, V K , REDDANNA, P , BHASKAR, M AND GOVINDAPPA, S. (1981*a*) Muscle metabolism of freshwater fish, *Tilapia mossambica* (Peters), during acute exposure and acclimation to sublethal acidic water *Can J Zool* **59**, 1909–1915

MURTHY, V K , REDDANNA, P AND GOVINDAPPA, S (1981*b*) Hepatic carbohydrate metabolism in *Tilapia mossambica* (Peters) acclimated to low environmental pH *Can J Zool* **59**, 400–404

NEVILLE, C M (1979) Sublethal effects of environmental acidification on rainbow trout (*Salmo gairdneri*) *J Fish Res Bd Can* **36**, 84–87

OGAWA, S AND LEE, T M (1984) The relation between the internal phosphorylation potential and the proton motive force in mitochondria during ATP synthesis and hydrolysis *J biol Chem* **259**, 10004–10011

OGAWA, S , ROTTENBERG, H , BROWN, T R , SHULMAN, R G , CASTILLO, C L AND GLYNN P (1978) High-resolution ^{31}P nuclear magnetic resonance study of rat liver mitochondria *Proc natn Acad Sci U S A* **75**, 1796–1800

STUART, S AND MORRIS, R (1985) The effects of season and exposure to reduced pH (abrupt and gradual) on some physiological parameters in brown trout (*Salmo trutta*) *Can J Zool* **63**, 1078–1083

TAM, W H , FRYER, J N , ALI, I , DALLAIRE, M R AND VALENTINE, B (1988) Growth inhibition, gluconeogenesis, and morphometric studies of the pituitary and interrenal cells of acid-stressed brook trout (*Salvelinus fontinalis*) *Can J Fish aquat Sci* **45**, 1197–1211

THOMA, W J AND UGURBIL, K (1988) pH and compartmentation of isolated perfused rat liver studied by ^{31}P and ^{19}F NMR *NMR Biomed* **1**, 95–100

ULTSCH, G R , OTT, M E AND HEISLER, N (1981) Acid–base and electrolyte status in carp (*Cyprinus carpio*) exposed to low environmental pH *J exp Biol* **93**, 65–80

VAN DEN THILLART, G , KÖRNER, F , VAN WAARDE, A , ERKELENS, C AND LUGTENBURG, J (1989*a*) A flow-through probe for *in vivo* ^{31}P-NMR spectroscopy of unanesthetized aquatic vertebrates at 9 4 Tesla *J magn Reson* **84**, 573–579

VAN DEN THILLART, G VAN WAARDE, A , DOBBE, F AND KESBEKE, F (1982) Anaerobic metabolism of goldfish, *Carassius auratus* (L) Effects of anoxia on the measured and calculated NAD$^+$/NADH ratios in muscle and liver *J comp Physiol* **146**, 41–49

VAN DEN THILLART, G VAN WAARDE, A , MULLER, H J , ERKELENS, C , ADDINK, A AND LUGTENBURG J (1989*b*) Fish muscle energy metabolism measured by *in vivo* ^{31}P-NMR during anoxia and recovery *Am J Physiol* **256**, R922–R929

VAN WAARDE, A , VAN DEN THILLART, G , ERKELENS, C , ADDINK, A AND LUGTENBURG, J (1990) Functional coupling of glycolysis and phosphocreatine utilization in anoxic fish muscle An *in vivo* ^{31}P-NMR study *J biol Chem* **265**, 914–923

WENDELAAR BONGA, S E AND DEDEREN, L H T (1986) Effects of acidified water on fish *Endeavour* **10**, 198–202

WENDELAAR BONGA, S E , FLIK, G AND BALM, P H M (1987) Physiological adaptation to acid stress in fish *Ann Soc R Zool Belg* **117**, 243–254

WENDELAAR BONGA, S E , FLIK, G , BALM, P H M AND VAN DER MEIJ, J C A (1990) The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water *Cell Tissue Res* (in press)

WENDELAAR BONGA, S E , VAN DER MEIJ, J C A AND FLIK, G (1984) Prolactin and acid stress in the teleost *Oreochromis* (formerly *Sarotherodon*) *mossambicus* *Gen comp Endocr* **55**, 323–332

The means and variations of the measured pH values are expressed as mean ± S.D., following the recommendation of Boutilier and Shelton (for discussion see R.G. Boutilier and G. Shelton, 1980: The statistical treatment of hydrogen ion concentration and pH. J. exp. Biol. **84**, 335 - 339).

CHAPTER 3:

THE INFLUENCE OF GRADUAL WATER ACIDIFICATION ON THE ACID/BASE STATUS AND PLASMA HORMONE LEVELS IN CARP

ABSTRACT

Carp (Cyprinus carpio) fitted with arterial catheters were subjected to gradual water acidification (from pH 7.6 to 4.0 in 4h), and then monitored for 48 hours. The measured blood parameters showed little or no disturbance; there was no ionoregulatory failure, no hyperglycemia, no increase in plasma lactate, no hypoxemia, nor swelling of red blood cells. Only a slight transient decrease of plasma pH_e was found. Plasma catecholamine concentrations remained at control levels. The plasma cortisol concentration showed a transient increase and returned to control values 24 hours after the start of water acidification.

Our results contrast with literature data on carp where exposure to pH 4.0 caused severe blood electrolyte disturbance. We conclude that the rate of water acidification (gradual in our study as compared to acute in literature) rather than the pH level itself determines the early effects of water acidification.

P. van Dijk, G. van den Thillart, P. Balm and S. Wendelaar Bonga; Journal of Fish Biology (in press)

The physiological effects of acid water on fish have been studied intensively over the last 15 years (see McDonald, 1983a; Howells, 1984; Wood, 1989, for reviews). From these studies, it became clear that the key toxic mechanism of low water pH is disturbance of the electrolyte balance at the gills, and not internal acidosis. A net H^+ ion influx is dependent on the relative movements of strong cations and anions. Thus, as is discussed by Wood (1989), water acidification can only lead to internal acidosis as a result of ionoregulatory failure. The ionoregulatory failure may also trigger circulatory collapse which finally causes death (Wood & McDonald, 1982).

Using carp, Ultsch et al. (1981) found that two instant step changes in water pH (from 7.4 to 5.1 and from 5.1 to 4.0) resulted in declining plasma sodium and chloride concentrations and a progressive reduction of arterial pH. A further decrease of the pH to 3.5 finally led to the death of the animals within 24 hours. Other studies performed with various fish species led to similar results (see McDonald, 1983a; Howells, 1984; Wood, 1989, for reviews).

Using tilapia (Oreochromis mossambicus), Wendelaar Bonga et al. (1987) showed that an immediate drop in water pH to pH 4.0 (the rate of water acidification was 18 pH units per hour) can lead to substantial structural damage of the branchial epithelium. However, at a lower rate of water acidification (3.0 pH units per hour and lower), these effects were not seen. A lower rate of water acidification is more environmentally relevant because, in nature, water acidification is gradual, not immediate (Seip & Tollan, 1978; Henriksen et al., 1984). Thus, studies on fish experiencing gradual acidification may give better insight in the tolerance to acid water of the species concerned.

In the present study, we wanted to investigate whether the disturbances in electrolyte and acid/base balance reported by others after acute acidification would take place when the

acidification proceeded at a sufficiently low rate (0.9 pH unit per hour) to prevent epithelial damage. The parameters we measured included blood parameters such as arterial extracellular pH, total CO_2, CO_2 tension, O_2 tension, haematocrit, hemoglobin concentration,and plasma parameters such as lactate, glucose, sodium, chloride, potassium, and total calcium. In addition, plasma cortisol and catecholamine levels were measured.

MATERIALS AND METHODS

Carp, Cyprinus carpio, (300-700 g) of both sexes were obtained from the O.V.B., Lelystad, The Netherlands and were held for at least 6 months, in dechlorinated and well-aerated local tap water of 20 ± 1°C. The fish were kept on a 14 L/10 D photocycle and fed daily with commercial trout pellets.

At least three weeks before the start of the experiments the fish were transferred to 4 times diluted local tap water. For this, 1 vol of copper-free tap water was mixed with 3 vols of demineralized (by ion exchangers) water. The end concentrations were as follows: Na^+ 0.83, Cl^- 0.92, Ca^{2+} 0.68, K^+ 0.07, Mg^{2+} 0.16, NO_3^-0.03, HCO_3^-1.11, SO_4^{2-}0.29, SiO_2 0.06 mmol l^{-1}; $Al_{tot.}$ was below the detection level of 6.7 nmol l^{-1}. Water composition was similar to our earlier experiments (Balm, 1986; see also chapter 2). During the acclimation period and throughout the experiments, the fish were kept in this water at 20.0 ± 0.5°C. To acclimate the fish to the confinement conditions of the experimental period, they were kept in separate chambers (26x26x35 cm). In the acclimation chambers, the water pH was maintained at 7.6 by a pH stat device. No metal parts were used in this set-up. Because preliminary experiments showed that trout pellets cause too much pollution of the water, fish were fed daily with cichlid food in flake form (Lapis, Europet, Nürnberg, FRG); feeding was suspended at least 24 h before cannulation. Experiments were performed over the period

October - December.

For catheterization (Soivio et al., 1972, 1975), 48 h before the experiments, a fish was anaesthetized in a Tris-buffered (pH 7) 100 mg l^{-1} MS222 solution. During surgery the gills were irrigated with a well-aerated Tris-buffered 75 mg l^{-1} MS222 solution (18.0 ± 0.5°C). After operation the fish was placed in a narrow PVC chamber with constantly flowing aerated water (0.5 l $min.^{-1}$). A pH stat device (METROHM 605/614/655) titrating 1 mol l^{-1} NaOH was used to maintain the water pH at 7.6.

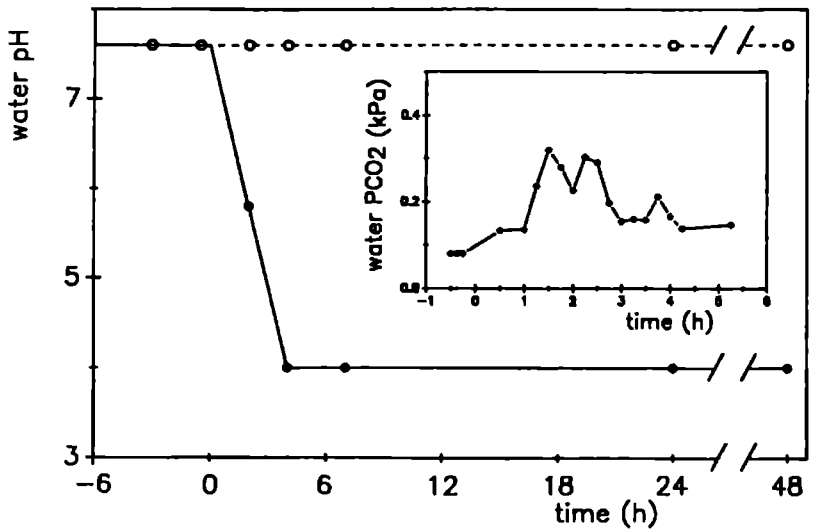


Fig. 1: Scheme of the experimental procedure. The water pH which was held constant at 7.6 for the control group (open circle), was gradually lowered for the acid group (filled circle). The dots mark the times blood samples were taken. Inset: water pCO_2 levels during the acidification period.

In this study there were two groups of fish: a control group (N=4) and an acidification group (acid group; N=5). In the acid group, blood samples were taken at three hours (t=-3h) and half an hour (t=-0.5h) before water acidification (see Fig. 1). Between t=0h and t=4h, the water pH was gradually lowered from pH 7.6 to pH 4.0, with 0.5 mol l^{-1} H_2SO_4 and a pH stat device. During this acidification period, the pCO_2 level of the water

was kept low by vigorous aeration. Further blood samples were taken at 2 hours (t=2h; pH=5.8), 4 hours (pH=4.0), 7 hours, 24 hours, and 48 hours after the start of the acidification. In the control group, blood samples were taken at the same intervals (see Fig. 1). The pH stat device was used throughout the experiment to keep the water pH at 7.6. The pH electrodes (Russel CTL/LCW for low conductivity) that were employed were specially designed to be used in solutions of low ionic strength.

Samples (750 µl) of whole blood were anaerobically withdrawn into ice-cold gas-tight Hamilton syringes via the dorsal aortic catheter. The blood was replaced by saline (Wolf, 1963). Blood samples were analyzed for arterial extracellular pH (pH_e), total CO_2 (TCO_2), CO_2 tension (pCO_2), O_2 tension (pO_2), haematocrit, hemoglobin concentration, and plasma levels of lactate, glucose, sodium, chloride, potassium, total calcium, adrenaline, noradrenaline, and cortisol.

To measure the water pCO_2, an air-tight syringe was filled with water from the set-up. After four minutes of constantly flushing a Radiometer bloodgas analyser (BMS3 M2), thermostatted to the experimental temperature and connected to a Radiometer PHM 71, the water pCO_2 was read. Arterial pH_e, pCO_2, and pO_2 were determined together by injecting 130 µl whole blood into the Radiometer bloodgas analyser. Total CO_2 was measured using a modified form of the method of Cameron (1971). A spike (100 µl 10 mM $NaHCO_3$), two standards (50 µl 10 mM and 50 µl 5 mM $NaHCO_3$), and two blood samples (50 µl each) were injected into the Cameron chamber successively. The two standards were used to calculate the TCO_2 of the blood samples, using a linear regression. In this calculation, a compensation was made for the volume driven out by each sample. From reference measurements the accuracy was found to be ± 0.1 mmol l^{-1}. Plasma lactate, glucose, and total calcium were measured using Sigma kits (St.Louis, MO). Plasma sodium, potassium, and chloride concentrations were measured using an auto analyser and a flame photometer.

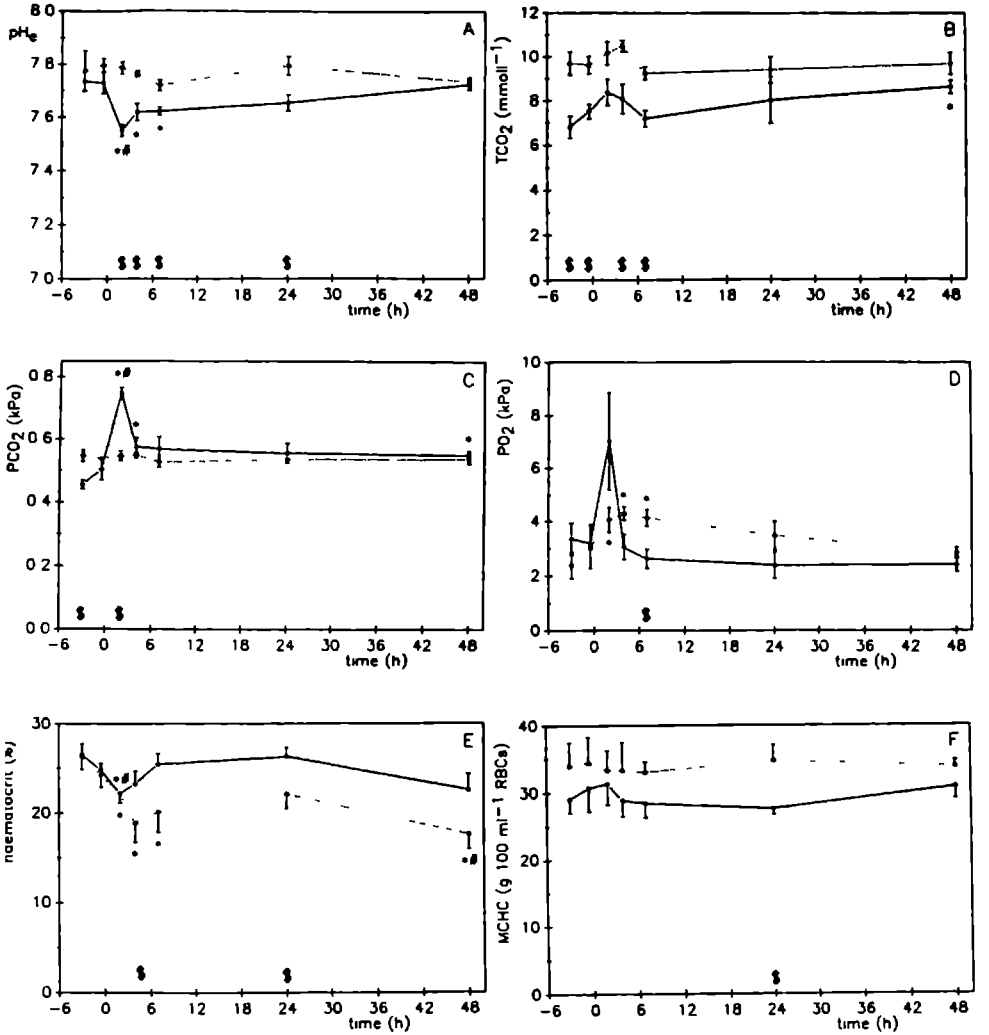


Fig. 2: The effect of gradual water acidification (see Fig. 1) on a) extracellular pH (pH_e), b) total CO_2, c) the partial pressure of carbon dioxide, d) the partial pressure of oxygen, e) haematocrit, and f) mean cellular hemoglobin concentration in the arterial blood of carp. Means ± S.E.M. Open circle, control group (N=4); filled circle, acid group (N=5);
*, signifies a significant difference (P≤0.05) within a treatment group from the corresponding t=-3h value;
#, signifies a significant difference (P≤0.05) within a treatment group from the corresponding t=-0.5h value;
$ signifies a significant difference (P≤0.05) between control and acid group at a particular sample time.

Immediately after blood sampling, 250 µl whole blood was added to 10 µl of a preservative mix (90 mg ml^{-1} EGTA plus 60 mg ml^{-1} glutathione in 3.6% KOH), plasma was separated by centrifigation, immediately frozen in liquid nitrogen, and stored at -80°C. In these plasma samples, catecholamines were measured, applying high performance liquid chromatography (De Potter et al., 1987). These measurements were performed by: Universitaire Instelling Antwerpen, 2610 Wilrijk, Belgium. Plasma cortisol was determined by radio-immunoassay (RIA) as described by Vecsei (1974) and De Man et al. (1980).

Data were expressed as means ± S.E.M. Statistical significance of differences ($P \leq 0.05$) was tested by the Mann-Whitney U-test.

RESULTS

In both the control and the acid groups there were no mortalities during the experiment. In the control group, significant changes in blood parameters were only a transient increase in blood pO_2 (Fig. 2d) and a decrease in haematocrit (Fig. 2e) due to blood sampling.

Water pCO_2 levels were monitored during the acidification period (Fig. 1). Before acidification, the water pCO_2 was 0.08 kPa, at t=1.5h the pCO_2 was maximal (0.32 kPa), at t=2h it was 0.23 kPa and at t=4h it was 0.16 kPa.

In the acid-treated fish, the pH_e (Fig. 2a) reached a minimum at t=2h (it decreased from 7.73 to 7.55). Two hours later, a slightly higher pH_e was measured, although the difference with the control group was still significant. After two days of acid exposure the blood pH was completely back to control level. In addition to a transient insignificant increase, the TCO_2 (Fig. 2b) tended to rise which led to a significant difference at t=48h. The increase in the blood pCO_2 (Fig. 2c) just after the start of the acidification coincided with a (not significant) increase in pO_2 (Fig. 2d). While the pO_2 was back at the control level at t=4h, the pCO_2 remained at a slightly elevated level

Time	-3h	-½h	2h	4h	7h	24h	48h
Plasma lactate (mmol l^{-1})							
acid group	1.26 ±.26	1.11 ±.08	0.80 ±.08	1.03 ±.12	1.82 ±.50	1.08 ±.10	1.15 ±.27
control group	0.96 ±.11	0.96 ±.10	1.05 ±.15	1.18 ±.22	1.17 ±.15	1.09 ±.05	1.45 ±.27
Plasma glucose (mmol l^{-1})							
acid group	1.4 ±.4	1.6 ±.9	0.8 ±.3	1.6 ±.6	1.0 ±.2	2.2* ±.7	1.5* ±.3
control group	1.2 ±.5	1.1 ±.4	1.1 ±.4	0.7 ±.3	0.5 ±.2	0.8 ±.3	0.4 ±.2
Plasma [Na^+] (mmol l^{-1})							
acid group	139.9 ± 2.1	142.2 ± 2.5	137.4 ± 5.4	147.1 ± 3.1	146.4 ± 5.2	138.0 ± 1.5	138.4 ± 5.6
control group	132.7 ± 3.8	139.4 ± 3.4	138.6 ± 5.1	142.9 ± 3.3	145.8 ± 7.0	142.9 ± 1.9	131.4 ± 4.9
Plasma [Cl^-] (mmol l^{-1})							
acid group	139.8 ± 5.1	135.8 ± 6.5	134.5 ± 4.6	144.0 ± 3.9	143.2 ± 9.0	139.7 ± 4.7	136.5 ±10.1
control group	123.1 ± 5.5	133.8 ± 7.7	130.8 ± 8.2	137.4 ± 0.9	140.1 ± 7.6	145.2 ± 4.9	135.8 ± 7.5
Plasma [K^+] (mmol l^{-1})							
acid group	2.68 ±.09	2.29* ±.22	2.36 ±.14	2.68 ±.26	3.63 ±.51	2.87 ±.10	4.41 ±1.3
control group	2.86 ±.40	3.03 ±.13	2 48 ±.14	2.41 ±.07	2.71 ±.24	3.10 ±.41	2.84 ±.17
Plasma [$Ca]_{tot}$ (mmol l^{-1})							
acid group	2.64 ±.20	2.07 ±.36	2.80 ±.24	2.40 ±.37	2.77* ±.20	2.86* ±.10	3.04 ±.20
control group	2.24 ±.24	2.09 ±.20	2.24 ±.20	-	1.96 ±.18	2.06 ±.03	2.43 ±.35

Table 1: The effect of gradual water acidification (between t=0h, pH 7.6 and t=4h, pH 4.0) on the plasma concentrations of lactate, glucose, sodium, chloride, potassium and total calcium in carp. Means ± S.E.M. +, signifies a significant difference ($P \leq 0.05$) between control and acid group at a particular sample time.

throughout the experiment. In the control group, the haematocrit (Fig. 2e) declined up to t=4h and then remained fairly constant until the end of the experiment. In the acid group, a similar initial reduction was seen, but the decrease of the haematocrit was less severe and more transient, and restoration followed after 7 hours. The mean cell hemoglobin concentration (MCHC; Fig. 2f) was constant within each group. Compared to the acid group, the control group had a slightly higher MCHC throughout the experiment. This difference between groups was significant at t=24h.

Plasma lactate, sodium, and chloride did not differ between the acid group and the control group (Table 1). At t=24h and t=48h, plasma glucose was significantly lower in the controls. In the acid group, plasma potassium was lower at t=-0.5h, and plasma total calcium was higher at t=7h and t=24h compared to the controls (Table 1).

No significant changes were observed in the plasma adrenaline (Fig. 3a) or noradrenaline concentrations (Fig. 3b). The values observed in the acid group remained close to the pre-experimental levels at t=-3h and t=-0.5h. As a reaction to the water acidification, the plasma cortisol levels in the acid group showed a transient increase (Fig. 3c). Between t=2h and t=4h, as the water pH decreased from 5.8 to 4.0, the cortisol concentration rose threefold and remained at this level up to t=7h. Seventeen hours later cortisol was similar to control level.

DISCUSSION

In this study, cannulated carp were exposed to sublethal levels of water acidity. Gradual acidification to pH 4.0 led to only minor transient changes in the measured blood parameters and did not cause any mortality in the two-day experimental period. Thus, carp are able to survive water of pH 4.0 for 48 hours without major physiological disturbances. In addition, in the first month after their return to the holding tanks, there

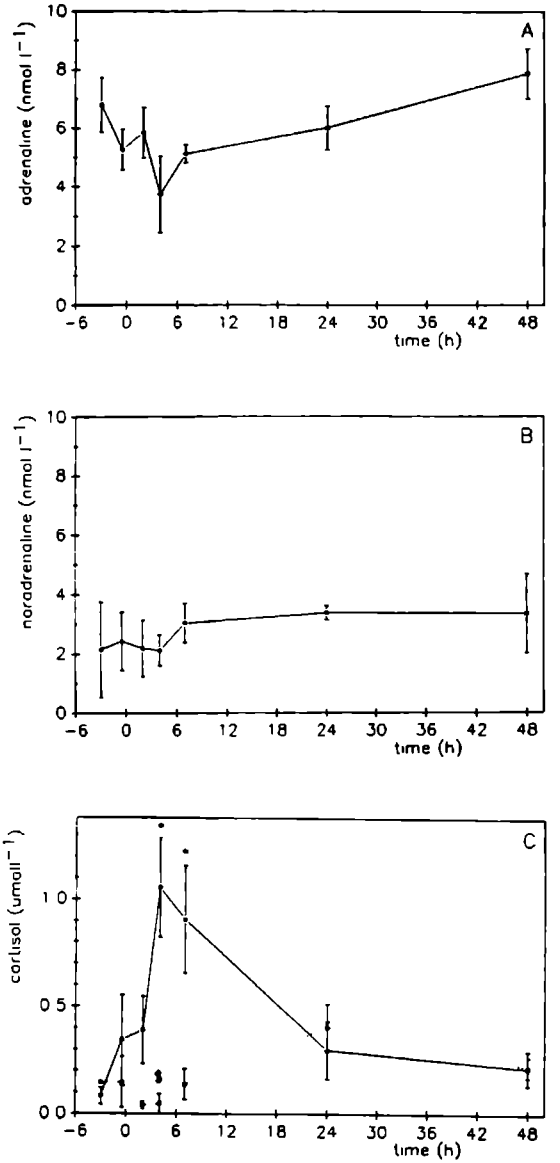


Fig. 3: The effect of gradual water acidification (see Fig. 1) on the plasma concentrations of a) adrenaline, b) noradrenaline, and c) cortisol in carp. Means ± S.E.M. Open circle, control group; filled circle, acid group; other details as in legend of Fig. 2. The catecholamine concentrations in the blood samples of the control fish were not determined.

was no mortality among the experimental fish.

These findings contrast with observations of Ultsch et al. (1981) on the same species. In those experiments, lowering of the water pH abruptly to 4.0 caused a decline of plasma ions and a progressive reduction of arterial blood pH. A 100% mortality within 24 hours was observed at pH 3.5. There are a few differences in experimental conditions and in the protocol between our study and the study of Ultsch and co-workers. The difference in acidification procedure is important. The immediate pH decrease Ultsch used contrasts with our procedure of gradual acidification.

Using tilapia, Wendelaar Bonga et al. (1987) found that a sudden drop in water pH (the rate of water acidification was equivalent to 18 pH units per hour), led to substantial structural damage of the branchial epithelium, which included the death of most of the chloride cells and severe loss of blood electrolytes. At a lower rate of water acidification (3 pH units per hour and lower), these effects were not seen. This is in agreement with a recent study on carp (Wendelaar Bonga, unpublished results), in which a slightly reduced plasma osmolarity after gradual acidification and a significantly lower plasma osmolarity after acute water acidification were found. Another factor which might have caused a much more severe disturbance in electrolyte status and acid/base balance in Ultsch' experiment is additional stress: The step change from pH 7.4 to 5.1 was reported to excite the animals. No obvious signs of excitement were observed during our acidification procedure. Balm (1986) showed that handling stress in tilapia in acid water impaired osmoregulation whereas the same type of handling had no effect under control conditions. The $[Ca]_{tot}$ in the experimental water was 4 to 5 times higher in Ultsch' experiment ($[Ca^{2+}]$ 2.8-3.5 mmol l^{-1}) than in our experiment. The marked depression in plasma pH_e found by Ultsch, in comparison with our results, is in agreement with the results of Wood (1989), who found that depressions in plasma pH were greatest at the highest water Ca^{2+} and were linearly reduced as Ca^{2+} drops. High water calcium levels are known to be protective against ionoregulatory

disturbances (McDonald, 1983b). Thus, in the study of Ultsch and co-workers, the ionoregulatory disturbances were substantial despite the high water $[Ca]_{tot}$.

To avoid hypercapnic conditions during the water acidification, we used a four compartment system, which was constantly vigorously aerated. To check if our set-up was successful in keeping water pCO_2 at control level, it was monitored during the experiment. Despite our precautions, the water pCO_2 rose and reached a maximum at t= 1.5h of 0.32 kPa. Claiborne & Heisler (1984) found in carp that mild hypercapnia for 2 hours ($pCO_2 \approx 1.0$ kPa) increased arterial blood pCO_2 (from 0.64 to 1.5 kPa), decreased plasma pH (from 7.87 to 7.63) and elevated plasma $[HCO_3^-]$ (from 14 to 16.5 mmol l^{-1}). So we assume that the increased blood pCO_2 of 0.25 kPa at t=2h was mainly due to the elevated water pCO_2 at this point of time. Possibly, at least part of the decrease of the blood pH_e had the same cause. The increased blood pCO_2 was not caused by hypoventilation since water acidification caused a transient rise in oxygen uptake in carp (P. van Dijk, G. van den Thillart and S. Wendelaar Bonga, unpublished results).

Erythrocyte recruitment due to spleen contraction is probably responsible for the less pronounced decrease in haematocrit (between t=-0.5h and t=48h) in the acid group compared to the control group. These new RBCs obviously did not have an effect on the mean cell hemoglobin concentration. The MCHC was constant throughout the experiment, also indicating the absence of red cell swelling or shrinkage. Similar to the spleen contraction, the red cell swelling is under adrenergic control. *In vitro* results in carp (Salama & Nikinmaa, 1988) have shown that the adrenergic-induced red cell swelling only occurred when either the plasma pH was below 7.5 at normal pO_2 or when at pH 7.5 the pO_2 of the incubation was decreased to 30 mm Hg or below. In the present study these conditions were never met, which explains the absence of red blood cell swelling.

The measured plasma catecholamine concentrations are at the upper end of the range known from the literature for cannulated fish at rest (Ristori and Laurent, 1985; Primmett *et al.*, 1986;

Butler et al., 1986, 1989). Even though the adrenaline level is relatively high, we do not take this as an indication for stress, since the concentration of cortisol, an other stress hormone, is not elevated before the start of the acidification. The values at t=-3h and t=-0.5h are comparable to results from other studies on resting fish (Edwards et al., 1987, Goss & Wood 1988, Witters, 1990).

The catecholamines remained at control levels throughout the experiment. A rise of catecholamines is typically associated with the alarm phase of the stress response. The absence in our experiment is an indication that our experimental protocal was not very stressful for the animals. It could be possible, however, that our sampling protocol did not detect a transient rise in adrenaline and/or noradrenaline. Tang and Boutilier (1988) found that plasma catecholamine levels peaked immediately after exhaustive exercise in rainbow trout, and then decreased rapidly over a subsequent 40 minute recovery period. Boutilier et al. (1986) suggested that in rainbow trout, plasma catecholamines increase in response to the rate of decrease of blood pH. We found a moderate decrease in pH_e of 0.2 units. In teleosts, due to the Bohr and Root effect, an acidosis can cause hypoxemia. Perry et al. (1989) demonstrated that in rainbow trout, hypoxemia rather than blood acidosis is the proximate stimulus causing the release of catecholamines into the circulation, during acute hypercapnic acidosis. From our data it cannot be expected that hypoxemia occurred since measured pH_e values are too high to cause a Root effect (Pelster and Weber, 1990), and the pO_2 of the blood did not decrease at any point. From these observations, we conclude that the plasma catecholamine concentration must have remained at control levels throughout the experiment. Interestingly, in rainbow trout, acute water acidification increased plasma catecholamine concentrations (Ye et al., 1991), but gradual water acidification (from pH 6.8 to 5.0 in 3 to 4 hours) did not cause any change in these hormones (Witters, 1991). It seems plausible to view the acute form of water acidification as an additional stressor, which, in combination with the low pH, causes the

plasma catecholamine concentrations to rise. Even though plasma catecholamines remained at control level in our experiments, we cannot exclude other factors, such as changes in turnover rate of catecholamines, to have a regulatory role. We have some evidence for tilapia (P. Balm, P. van Dijk, G. van den Thillart and S. Wendelaar Bonga, unpublished results) that fish exposed to acid water (3 days at pH 4.0) have a lower turnover rate of catecholamines.

Although gradual reduction of the water pH did not noticeably influence electrolyte and acid/base balance of the extracellular fluid, exposure to acid water was experienced as a stressor. This is indicated by the transient peak in the plasma cortisol concentration, which is typical for the primary response of fish to stress, including acid exposure. Goss and Wood (1988) found in rainbow trout that exposure to pH 4.8 caused a transient increase in plasma cortisol concentration. Cortisol was back at control level after one day, very similar to what was found in this study. Combined acid and aluminium stress (pH = 4.8, Al = 112 $\mu g\ l^{-1}$), however, caused permanent high plasma cortisol levels until death (Goss and Wood, 1988). Chronic elevation of plasma cortisol levels are known to have a negative effect on a wide range of physiological processes, including the immune system (Mazeaud and Mazeaud, 1981; Barton and Iwama, 1991).

The return of the plasma cortisol concentration to the control level does not necessarily imply that cortisol dynamics returned to control values. Balm (1986) suggested that after two days of acid exposure, the turnover rate of cortisol in tilapia had increased. He came to this conclusion because he found an increased release of cortisol by the headkidneys even though plasma cortisol concentrations were similar to those of the controls. Cortisol stimulates the Na^+-dependent ATPase activity of the chloride cells, which promotes active Na^+-uptake (Mayer-Gostan *et al.*, 1987). In carp, acute acid exposure was demonstrated to lead, via a net sodium efflux, to a reduced plasma sodium concentration (Ultsch *et al.*, 1981). The fact that in the present study plasma sodium was not decreased while

plasma cortisol was elevated might mean that a rise in sodium efflux was compensated for by an increase in active sodium uptake.

REFERENCES

Balm, P.H.M. (1986). Osmoregulation in teleosts by cortisol and prolactin. Adaptation to low pH environments. Ph.D. thesis, University of Nijmegen, The Netherlands.

Barton, J.A., & Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Boutilier, R.G., Iwama, G.K., & Randall D.J. (1986). The promotion of catecholamines release in rainbow trout (Salmo gairneri), by acute acidosis: interactions between red cell pH and haemoglobin oxygen-carrying capacity. Journal of Experimental Biology 123, 145-157.

Butler, P.J., Metcalfe J.D., & Ginley, S.A. (1986). Plasma catecholamines in the lesser stotted dogfish and rainbow trout at rest and during different levels of exercise. Journal of Experimental Biology 123, 409-421.

Butler, P.J., Axelsson, M., Ehrenström, F., Metcalfe, J.D., & Nilsson, S. (1989). Circulating catecholamines and swimming performance in the atlantic cod, Gadus morhua. Journal of Experimental Biology 141, 377-387.

Cameron, J.N. (1971). Rapid method of determination of total carbon dioxide in small blood samples. Journal of Applied Physiology 31, 632-634.

Claiborne, J.B., & Heisler, N. (1984). Acid-base regulation and ion transfer in the carp (Cyprinus carpio) during and after exposure to environmental hypercapnia. Journal of Experimental Biology 108, 25-43.

De Man, A.J.M., Hofman, J.A., Hendriks, Th., Rosmalen, F.M.A. Ross, H.A., & Benraad, Th.J. (1980). A direct radio-immunoassay for plasma aldosterone: significance of

endogenous cortisol. Netherlands Journal of Medicine 23, 79-83.

De Potter, W.P., Coen, E.P., & De Potter R.W. (1987). Evidence for the coexistence and co-release of (met)enkephalin and noradrenalin from sympathetic nerves of the bovine vas deferens. Neuroscience 3, 855-866.

Edwards, D., Brown, J.A., & Whitehead, C. (1987). Endocrine and other physiological indicators of acid stress in the brown trout. Annales de la Societe Royale Zoologique de Belgique 117, 331-342.

Goss, G.G., & Wood, C.M. (1988). The effects of acid and acid aluminium exposure on circulating plasma cortisol levels and other blood parameters in the rainbow trout, Salmo gairdneri. Journal of Fish Biology 32, 63-76.

Henriksen, A., Skogheim, O.K., & Rosseland, B.O. (1984). Episodic changes in pH and aluminium-speciation kill fish in a norwegian river. Vatten 40, 255-260.

Howells, G.D. (1984). Fishery decline: mechanisms and predictions. Philosophical Transactions of the Royal Society of London B305, 529-547.

Mayer-Gostan, N., Wendelaar Bonga, S.E., & Balm, P.H.M. (1987). Mechanisms of hormone action on gill transport. In Vertebrate Endocrinology: Fundamentals and Biomedical Implications. Vol.2. Regulation of water and Electrolytes (ed. Pang, P.K.T., & Schreibman, M.P.), pp. 211-238. San Diego: Academic Press.

Mazeaud, M.M., & Mazeaud, F. (1981). The role of catecholamines in the stress response of fish. In Stress and fish (ed. Pickering, A.D.) London: Academic Press.

McDonald, D.G. (1983a). The effects of H^+ upon the gills of freshwater fish. Canadian Journal of Zoology 61, 691-703.

McDonald, D.G. (1983b). Interaction of environmental calcium and low pH on the physiology of the rainbow trout, Salmo gairdneri. I. Branchial and renal net ion and H^+ fluxes. Journal of Experimental Biology 102, 123-140.

Pelster, B., & Weber, R.E. (1990). Influence of organic phosphates on the root effect of multiple fish

haemoglobins. Journal of Experimental Biology 149, 425-437.

Perry, S.F., Kinkead, R., Gallaugher, P., & Randall, D.J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (Salmo gairdneri). Respiration Physiology 77, 351-364.

Primmett, D.R.N., Randall, D.J., Mazeaud, M.M., & Boutilier, R.G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout during exercise. Journal of Experimental Biology 122, 139-148.

Ristori, M.T., & Laurent; P. (1985). Plasma catecholamines and glucose during moderate exercise in the trout: comparison with bursts of violent activity. Experimental Biology 44, 247-253.

Salama, A., & Nikinmaa, M. (1988). The adrenergic responses of carp (Cyprinus carpio) red cells: effects of pO_2 and pH. Journal of Experimental Biology 136, 405-416.

Seip, H.M., & Tollan, A. (1978). Acid precipitation and other possible sources for acididification of rivers and lakes. Science of the Total Environment 10, 253-270.

Soivio, A., Westman, K., & Nyholm, K. (1972). Improved method of dorsal aorta catherization: haematological effects followed for three weeks in rainbow trout (Salmo gairdneri). Finnish fisheries research I, 11-21.

Soivio, A., Nyholm, K., & Westman, K. (1975). A technique for repeated sampling of blood of individual resting fish. Journal of Experimental Biology 63, 207-217.

Tang, Y., & Boutilier, R.G. (1988). Correlation between catecholamine release and degree of acidotic stress in trout. American Journal of Physiology 255, R395-R399.

Ultsch, G.R., Ott, M.E., & Heisler, N. (1981). Acid-base and electrolyte status in carp (Cyprinus carpio) exposed to low environmental pH. Journal of Experimental Biology 93, 65-80.

Vecsei, P. (1974). Glucocorticoids: cortisol, corticosterone and compound S. In Methods of Hormone Radioimmunoassay (eds. B.M. Baffe & H.R. Behrmar) p.393. Academic Press, New York.

Wendelaar Bonga, S.E., Flik, G., & Balm, P.H.M. (1987).

Physiological adaptation to acid stress in fish. Annales de la Societe Royale Zoologique de Belgique 117, 243-254.

Witters, H. (1990). Physiological study on the waterbug, Corixa punctata and the trout, Oncorhynchus mykiss: effects of aluminium in acid water. Ph.D. thesis, University of Antwerp, Belgium.

Witters, H.E., van Puymbroeck, S., & Vanderborght O.L.J. (1991). Adrenergic response to physiological disturbances in rainbow trout, Oncorhynchus mykiss, exposed to aluminium at acid pH. Canadian Journal of Fisheries and Aquatic Sciences 48, 414-420.

Wolf, K. (1963). Physiological salines for fresh-water teleosts. Progressive Fish-Culturist 25, 135-140.

Wood, C.M., & McDonald, D.G. (1982). Physiological mechanisms of acid toxicity to fish. In Acid Rain/Fisheries (ed. R.E. Johnson), pp. 197-226. Bethesda: American Fisheries Society.

Wood, C.M. (1989). The physiological problems of fish in acid waters. In Acid Toxicity and Aquatic animals (ed. R. Morris, E.W. Taylor, D.J.A. Brown, & J.A. Brown), pp. 125-152. Cambridge: Cambridge University Press.

Ye, X., Randall, D.J., & He, X. (1991). The effect of acid water on oxygen consumption, circulating catecholamines and blood ionic and acid-base status in rainbow trout (Salmo gairdneri, Richardson). Fish Physiology and Biochemistry 9, 15-21.

CHAPTER 4:

IS THERE A SYNERGISTIC EFFECT BETWEEN STEADY STATE EXERCISE AND WATER ACIDIFICATION IN CARP?

ABSTRACT

Carp (Cyprinus carpio) fitted with arterial catheters were trained to swim in a swim tunnel. While constantly swimming at two body lengths per second, they were subjected to gradual water acidification (from pH 7.6 to 4.0 in 4 hours), and monitored subsequently for 24 hours. It was hypothesized that exercise would expose the gills to a larger extent and that consequently the effect of water acidification would be greater in exercising than in resting carp. In contrast to our earlier study with resting carp (see chapter 3), we found a constant decline of plasma pH and plasma Na^+ and Cl^- concentration, indicating ionoregulatory failure. While plasma catecholamines remained at control levels, the plasma cortisol showed a three- to fourfold increase over the first 7 hours. This was followed by a slow decline. We conclude that there is a synergistic effect between moderate exercise and gradual water acidification in carp.

P. van Dijk, G. van den Thillart and S. Wendelaar Bonga; Journal of Fish Biology (in press).

In resting fish, the key toxic mechanism of low water pH is disturbance of electrolyte balance at the gills (see McDonald, 1983; Howells, 1984; Wood, 1989, for reviews). Ultsch et al. (1981) found severe ionoregulatory failure in carp in response to two instant step changes in water pH (from 7.4 to 5.1 and from 5.1 to 4.0). However, when the same species was exposed to pH 4.0, after gradual water acidification (0.9 pH units per hour), we did not find any electrolyte imbalance or much disturbance of the acid/base status (see chapter 3). Thus, the rate of acidification rather than the pH level of the water itself determined the response to water acidification in these experiments, and this apparently leads to overestimation of the deleterious effects of acid water. Nevertheless, the absence of detectable disturbances in the extracellular fluid does certainly not exclude that water of pH 4.0 acts as a stressor on carp. The results may reflect that, at pH 4.0, the threshold for ionoregulatory disturbance has not been reached, unless the low water pH is accompanied by an additional stressor such as a high rate of water acidification. To test this hypothesis, we examined the physiological performance of acid exposed fish using steady state exercise as a second stressor, and compared the effects with our earlier results on resting acid exposed carp.

There is evidence that acid exposure and strenuous exercise serve as potentiating or additive stressors. In fish during steady state exercise, blood acid/base, metabolite and electrolyte status as well as plasma catecholamine levels were largely unchanged when compared to the resting state (Stevens and Randall, 1967; Van den Thillart et al., 1983; Ristori and Laurent, 1985). A few minutes of exhaustive exercise, however, leads to a profound blood acidosis, to an elevated lactate plasma concentration, to increases in plasma ions and catecholamine levels (Ristori and Laurent, 1985; Turner et al., 1983; Van Dijk and Wood, 1988; Wood and Perry, 1985). In their 7-day lethality test on acid toxicity Graham and Wood (1981)

found that exercise until exhaustion shortened the survival time in fingerling trout. Similarly Graham et al. (1982) found in rainbow trout in soft water that acid exposure exacerbated most of the post-exercise disturbances and caused a doubling of mortality. More recently, Butler et al. (1992) found that critical swimming speed of brown trout was depressed at pH 4.5 (15°C) and at pH 4.0 (5°C). Similarly, Ye and Randall (1991) found a reduction in critical swimming velocity of rainbow trout in acid water (pH 4.0 and 5.0). They also found that the restoration of the blood acid/base balance after exhaustive exercise in acid water was impaired. In contrast, during steady state exercise, fish do not have the anaerobic load or the additional stress of handling of chased fish, and thus may be expected to deal better with acid exposure than fish during strenuous exercise. Still, in comparison to resting fish, their gills are more exposed to the environment because of the increased branchial blood perfusion and lamellar recruitment associated with exercise (Jones and Randall, 1978). Therefore, more ionic losses and a greater strain on ionic and osmoregulatory processes could be expected. This is confirmed by results of Gonzalez and McDonald (1992) with rainbow trout, who found that the sodium losses across the gills increased substantially whenever oxygen consumption increased. Altogether the impact of water acidification can be predicted to be larger during exercise than at rest.

For this reason we investigated the combined effects of acid exposure and steady state exercise on carp. We determined the blood metabolite, electrolyte and acid/base status, as well as the blood levels of the typical stress hormones, catecholamines and cortisol. In carp at rest, gradual water acidification did not cause many changes in these blood parameters (see chapter 3). Is the same true for cannulated carp during moderate exercise or is there a synergistic effect between acid exposure and steady state exercise? The answer to this question has environmental relevance because of the impact that reduced physical performance will have on the survival prospects of fish populations.

Carp, Cyprinus carpio (body mass 1,099 ±102 g; length 33.64 ± 0.75 cm), were obtained, maintained, and handled as described in chapter 3. At least 3 weeks prior to, and during the experiments the fish were held at 20.0 ± 0.5°C in well aerated fresh water containing the following: Na^+ 0.83, Cl^- 0.92, Ca^{2+} 0.68, K^+ 0.07, Mg^{2+} 0.16, NO_3^- 0.03, HCO_3^- 1.11, SO_4^{2-} 0.29, SiO_2 0.06 mmol l^{-1}; $Al_{tot.}$ was below the detection level of 6.7 nmol l^{-1}. Experiments were performed in July and August.

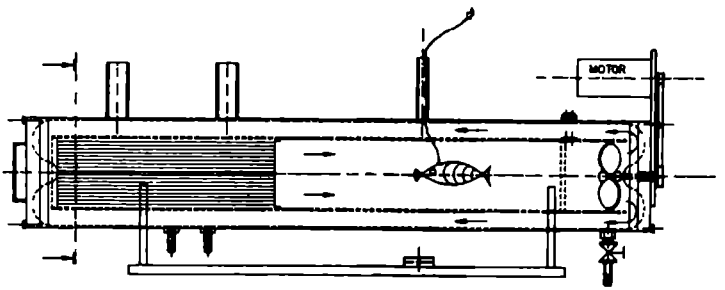


Fig. 1: Schematic drawing of the swim tunnel. The water in the double cylinder tube was circulated by a specially designed rotor, which caused a flow in the inner cylinder in the range of 0 - 2.5 m s^{-1}. A 0.8 m long honeycomb-shaped matrix was used to reduce turbulance. The free swimming space for the fish was 0.8 m long. An electrified stainless steel grid was situated behind the fish to prevent it from resting during the experiment. The water in the swim tunnel was replaced at a rate of 10 l min^{-1} from a 300 l tank filled with thermostatted (20.0 ± 0.5°C), air-saturated water. A pH-stat device was used to regulate the water pH. To prevent growth of micro-organisms, the water in the system was constantly pumped through glass tubes that were irradiated by two UV-lamps.

The swim tunnel (see Fig. 1) was specially developed for our large size fish. The water flow inside the swim tunnel was checked and calibrated, over the range of 0 - 2 m s^{-1} with a laser-doppler technique, by the Institute for Hydrodynamics, Technical University of Delft, The Netherlands. The flow rates

measured over the tube diameter did not deviate for more than 2% for each 1 cm^2.

After implantation of the dorsal aortic cannulae (Soivio et al., 1972, 1975), the loose end of the catheter was attached to the dorsal fin and the fish, which was still anaesthetised, was placed in the swim tunnel. As soon as the fish woke up, the water flow inside the swim tunnel was started and the fish was forced to swim at a low speed (0.8 BL s^{-1}; BL = body length). The next day the fish was trained to swim at moderate speed (1.5 - 2.5 BL s^{-1}). Overnight (17.00 h - 8.30 h) the swimming speed was 1.5 BL s^{-1} and the next morning it was increased to 2.0 BL s^{-1}, at which speed the fish swam throughout the experiment without any apparent difficulty. Between 1.0 and 1.5 hour after the change to 2.0 BL s^{-1}, the first blood sample was taken (t=-3h). The second sample was taken half an hour (t=-0.5h) before the start of the water acidification. From t=0h to t=4h the water pH was gradually lowered from pH 7.6 to 4.0, using 0.5 mol l^{-1} H_2SO_4 and a pH-stat device. Measurements showed that the partial pressure of carbon dioxide of the water in the swim tunnel did not increase during this acidification period. Further blood samples were taken two hours (t=2h; pH=5.8), 4 hours (pH=4.0), 7 hours and 24 hours after the start of the acidification. This experiment was performed with 5 animals (acid group; N=5). In the control group (N=2), blood samples were taken at identical times. Here the pH-stat device kept the water pH at 7.6 with 1.0 mmol l^{-1} NaOH.

Samples (750 µl) of whole blood were drawn anaerobically into ice-cold gas-tight Hamilton syringes via the dorsal aortic catheter. The blood was replaced by saline (Wolf, 1963). Blood samples were analysed for arterial extracellular pH (pH_e), total CO_2 (TCO_2), CO_2 tension (pCO_2), O_2 tension (pO_2), haematocrit, hemoglobin concentration, and plasma levels of lactate, glucose, sodium, chloride, potassium, total calcium, adrenaline, noradrenaline, and cortisol. The analytical methods employed have been described in chapter 3.

Data were expressed as means ± S.E.M. for the acid group (N=5) and as means for the control group (N=2). Significant

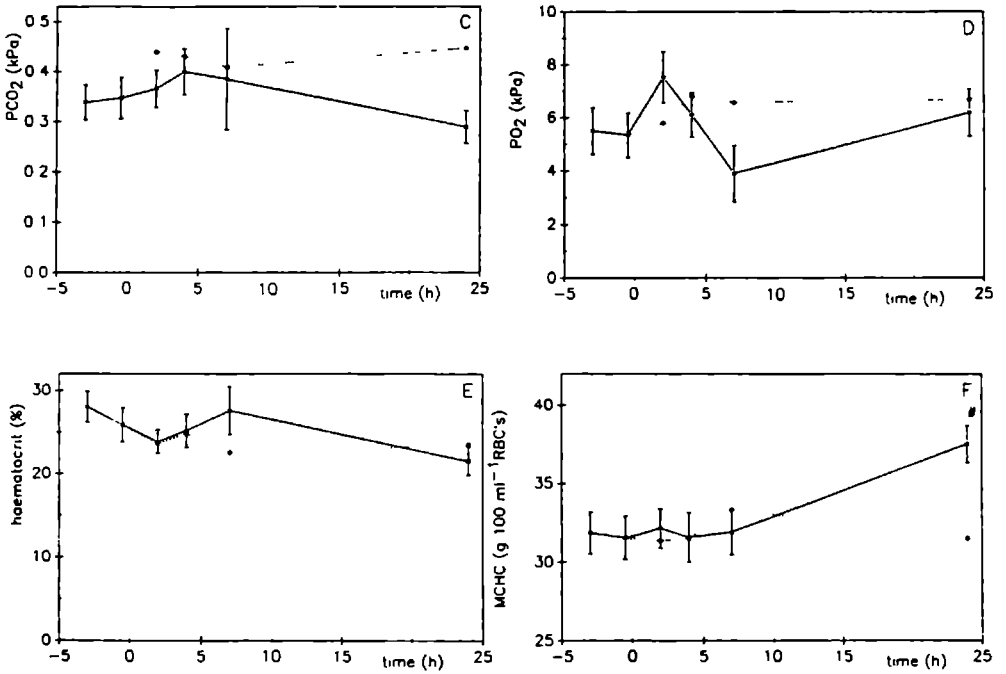


Fig. 2: The combined effect of acid exposure and steady state exercise on : a) the extracellular pH, b) total CO_2, c) the partial pressure of carbon dioxide, d) the partial pressure of oxygen, e) haematocrit, f) mean cell hemoglobin concentration in the arterial blood of carp. Throughout the experiment the fish swam at 2 BL s^{-1}. Water acidification took place between t=0h (pH 7.6) and t=4h (pH 4.0). Open circle: control group (N=2), means; filled circle: acid group (N=5), means ± S.E.M.;
*: a significant difference (P≤0.05) within the acid group from the t=-3h value;
#: a significant difference (P≤0.05) within the acid group from the t=-0.5h value.

differences ($P \leq 0.05$) were tested by the Mann-Whitney U-test. Because the treatment of the two groups was identical until the start of the water acidification, their data were combined at t=-3h and at t=-0.5h for the sake of clarity of illustrations.

RESULTS

Both in the control group and in the acid group there were no mortalities during the experiments.

Water acidification combined with steady state exercise (2 BL s^{-1}) caused a gradual decrease in arterial blood pH, which was significant at t=24h (Fig.2a). In the acid group, no significant changes in blood TCO_2 (Fig. 2b) and pCO_2 (Fig. 2c) were found. The blood pO_2 level (Fig. 2d) showed a zigzag. These deviations, however, were not significant. At t=24h, a decrease in haematocrit (due to blood sampling) and an increase of the mean cell hemoglobin concentration occurred (Fig. 2e and 2f).

In Fig. 3a it is shown that plasma lactate remained at a relatively low level. Just after the start of the water acidification (at t=2h), there was a decrease in plasma glucose concentration, which was significant in comparison with the t=-3h sample. At t=24h there was a slight hyperglycemia (Fig. 3b). Over the first 7 hours of acid exposure, no significant effect was observed on $[Na^+]$ and $[Cl^-]$; however, at t=24h the $[Na^+]$ and the $[Cl^-]$ were decreased to the same extent (Fig. 4a and 4b). This decrease was only significant for plasma chloride. There were no significant changes in the plasma potassium (Fig. 4c) and total calcium (Fig. 4d) concentrations.

Water acidification combined with steady state exercise did not cause any significant changes in the plasma catecholamine levels (Fig. 5a and 5b). The plasma cortisol concentration (Fig. 5c) rose after the start of the acidification procedure and was at its maximum at t=7h, where the levels were 3 to 4 times higher than in the controls. Thereafter plasma cortisol decreased and at t=24h it was not significantly different from the control values.

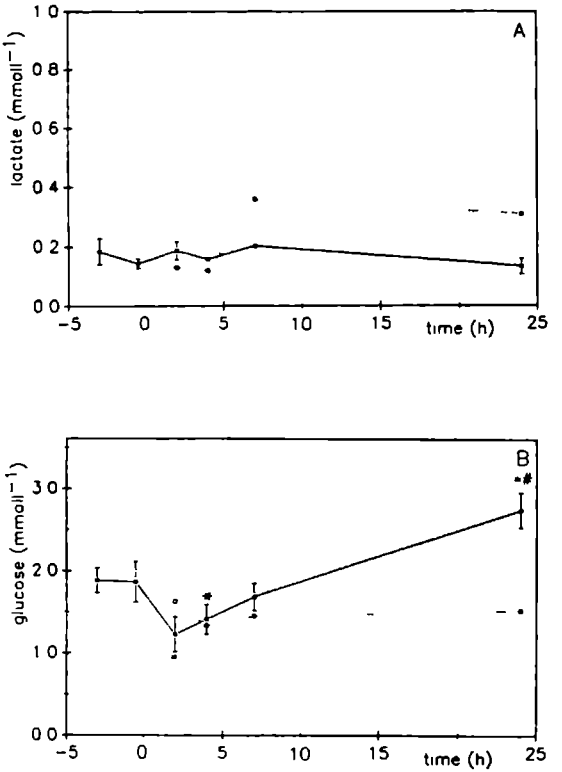


Fig. 3: The combined effects of acid exposure and steady state exercise on the plasma concentrations of a) lactate, and b) glucose in carp. Throughout the experiment the fish swam at 2 BL s^{-1}. Water acidification took place between t=0h (pH 7.6) and t=4h (pH 4.0). Open circle: control group (N=2), means; filled circle: acid group (N=5), means ± S.E.M.; other details as in legend of Fig. 2.

DISCUSSION

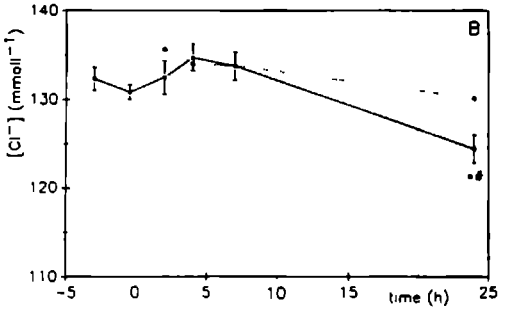
In literature, there is some evidence that strenuous exercise and acid exposure serve as potentiating or additive stressors. In their lethality test on acid toxicity, Graham and Wood (1981) found that exhaustive exercise decreased the

survival time in fingerling trout. Their protocol involved handling the fish immediately after exhaustion. Handling in combination with water acidification impairs the osmoregulation whereas handling has no measurable effect in neutral water (Balm, 1986). Another study that dealt with acid exposure in combination with strenuous exercise is that of Graham et al. (1982). Although the authors reported that the acid exposed fish probably performed less swimming activity than the control fish, their results nevertheless showed that, in soft water, acid exposure greatly aggravated most of the post-exercise disturbances and caused a doubling of mortality.

At t=24h we found a significant decrease in arterial blood pH. In our study on carp at rest, this effect did not occur (see chapter 3). Even 48 hours at pH 4.0 did not significantly change the pH_e in resting carp. This difference between our two studies supports the hypotheses, expressed in the Introduction, that the impact of water acidification on fish is larger during moderate exercise than at rest.

Compared with our results on carp at rest, we found significantly higher blood oxygen tensions in exercising carp, both at t=-3h and at t=-0.5h (i.e., before water acidification). With coho salmon, Van den Thillart et al. (1983) did not find any difference in arterial blood oxygen tension before and after one hour swimming at 80% of their critical speed. In resting carp,however, the arterial pO_2 is much lower than in salmonids, leaving more space for a possible increase. Increased ventilation and improved gill diffusion conductance during swimming are probably responsible for the observed rise in arterial pO_2.

The mean cell hemoglobin concentration measurements indicate that there was no red cell swelling. Neither was red cell swelling was neither found in our study with carp at rest. This absence of RBC swelling is in agreement with the in vitro results with carp of Salama and Nikinmaa (1988; see chapter 3 for discussion). In fact our present measurements indicate a significant red cell shrinkage after one day at pH 4.0. In tench, Jensen (1987) found red cell shrinkage in response to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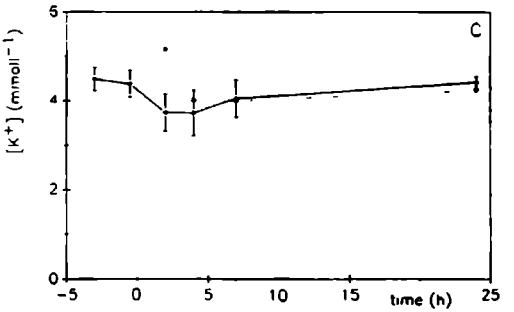
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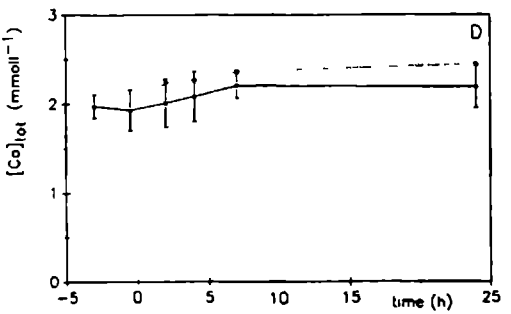
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adrenaline infusion. An electrolyte shift from the red bloodcells to the extracellular compartment, causing water movement in the same direction, might explain RBC shrinkage. It is not clear what caused this electrolyte shift since in our experiment we did not find an increase of plasma catecholamines.

Before water acidification, at t=-3h and t=-0.5h, plasma lactate was a factor 7 lower in this study, than in resting carp. Lactate measurements in a single body compartment with periodic sampling give little information about the dynamic equilibrium between production, metabolism, and fluxes between compartments. Anaerobic glycolysis and the resulting lactate acid does not occur during moderate exercise (see below). Evidently, the metabolic rate is higher during steady state exercise (2 BL s^{-1}) than in resting fish. Swimming is known to increase the oxidation rate of lactate (Van den Thillart, 1986). Bilinski and Jonas (1972) showed that rainbow trout heart and liver are capable of taking up and oxidizing exogenous lactate. Lactate is even a preferred substrate for the trout heart (Lanctin et al., 1980). Therefore, the lower plasma lactate concentration in this study must be due to an overall higher lactate oxidation rate.

Our exercise regimen did not cause an increase in the plasma lactate concentration. The absence of any such increase is a strong indication that our exercise regimen did not include burst-type exercise. As is generally accepted, red muscle fibers are specialized for long term, low-intensity exercise whereas white muscle fibers are involved in burst-type or high-intensity exercise (Bone, 1966). Steady state exercise by red muscle is fuelled by mitochondrial oxidation of pyruvate (derived from lactate, glucose or glycogen), fatty acids and ketone bodies.

Fig. 4: The combined effects of acid exposure and steady state exercise on the plasma concentrations of a) sodium, b) chloride, c) potassium, and d) total calcium in carp. Throughout the experiment the fish swam at 2 BL s^{-1}. Water acidification took place between t=0h (pH 7.6) and t=4h (pH 4.0). Open circle: control group (N=2), means; filled circle: acid group (N=5), means ± S.E.M.; other details as in legend of Fig. 2.

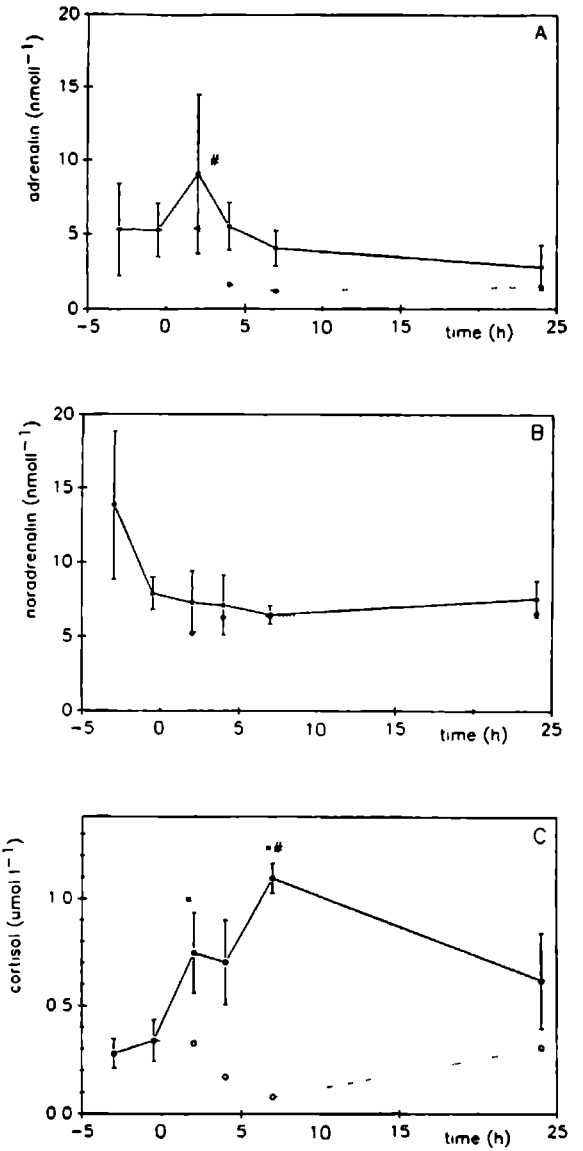

Fig. 5: The combined effects of acid exposure and steady state exercise on the plasma concentrations of a) adrenaline, b) noradrenaline, and c) cortisol in carp. Throughout the experiment the fish swam at 2 BL s^{-1}. Water acidification took place between t=0h (pH 7.6) and t=4h (pH 4.0). Open circle: control group (N=2), means; filled circle: acid group (N=5), means ± S.E.M.; other details as in legend of Fig. 2.

Burst-type exercise by white muscle depends on non-oxidative pathways such as phosphagen hydrolysis and anaerobic glycolysis. This type of exercise leads to an increased lactic acid concentration in the blood (Turner et al., 1983). From the unchanged lactate levels we conclude that our fish solely performed aerobic exercise. This is in agreement with Rome et al. (1984) who observed, in adult carp, that at 20°C the recruitment of white muscle fibers took place at swimming speeds between 2.0 and 2.5 Bl s^{-1}. Except for one fish in the acid group, we did not observe the burst-type of exercise. Shortly before the t=7h blood sample was taken, this fish started to show an irregular type of swimming: occasional drifting back with the water flow was followed by a short burst of intense swimming. Since this behaviour is quickly followed by fatique (Heap & Goldspink, 1986), we stopped the experiment with this fish.

After one day at pH 4.0, the plasma sodium and chloride concentrations were decreased to the same extent, which is an indication for ionoregulatory failure. At t=24h there was a slight hyperglycemia which, to some extent, compensated for the fall in plasma osmolarity, caused by the lower $[Na^+]$ and $[Cl^-]$ at this point of time. With carp at rest we did not find any changes in plasma ions or glucose. These differences between our two studies again support the idea of a larger impact of acid exposure during steady state exercise. In conclusion, in carp, moderate exercise or gradual water acidification alone, does not cause any changes in plasma glucose or plasma ions, whereas the two combined do cause ionoregulatory failure and hyperglycemia. Thus, we conclude that there is a synergistic effect between gradual water acidification (0.9 pH units per hour) and steady state exercise (2 BL s^{-1}) in carp.

The plasma adrenaline levels in moderately exercising animals were similar as in resting carp (see chapter 3), whereas the noradrenaline concentrations were about three times as high as the resting levels. In dogfish, Butler et al. (1986) observed an increase of adrenaline by a factor of 3.3 to 9 nM, and of noradrenaline by 2.3 to 32 nM, during spontaneous swimming. With

trout, Ristori and Laurent (1985) did not find any significant difference of these catecholamines between resting and moderately swimming (2.2 $BL \cdot s^{-1}$) animals. The concentrations of both amines ranged between 1 and 2 nM. Violent exercise, however, caused a significant increase in both parameters (Ristori & Laurent, 1985). Species differences might be responsible for the different results with trout and dogfish. Our results with carp indicate that moderate exercise causes a significant rise in noradrenaline, but not in adrenaline.

The shape of the cortisol curve in the present study shows similarity to that found in our earlier study with resting animals (see chapter 3). The peak values were about 1 µM in both studies. A transient increase of cortisol is typical for the primary response of fish to stress, including acid exposure (Goss & Wood, 1988).

In the present study no correlation between cortisol and glucose concentration was found. Both cortisol and adrenaline have been demonstrated to have hyperglycaemic effects on fish (Mazeaud et al., 1977). As was discussed by Morata et al. (1982) the endocrine regulation of glucose plasma levels varies between species. The present results indicate that in carp the plasma levels of neither cortisol nor adrenaline induce hyperglycemia.

REFERENCES

Balm, P.H.M. (1986). Osmoregulation in teleosts by cortisol and prolactin. Adaptation to low pH environments. Ph.D. thesis, University of Nijmegen, The Netherlands.

Bilinski, E., & Jonas, R.E.E. (1972). Oxidation of lactate to carbon dioxide by rainbow trout (Salmo gairdneri) tissues. Journal of Fisheries Research Board of Canada 29, 1467-1471.

Bone, Q. (1966). On the function of the two types of myotomal muscle fiber in elasmobranch fish. Journal of the Marine Biological Association of the United Kingdom 46, 321-349.

Butler, P.J., Metcalfe , J.D., & Ginley, S.A. (1986). Plasma

catecholamines in the lesser stotted dogfish and rainbow trout at rest and during different levels of exercise. Journal of Experimental Biology 123, 409-421.

Gonzalez, R.J., & McDonald, D.G. (1992). The relationship between oxygen consumption and ion loss in a freshwater fish. Journal of Experimental Biology 163, 317-332.

Goss, G.G., & Wood, C.M. (1988). The effects of acid and acid aluminium exposure on circulating plasma cortisol levelsand other parameters in the rainbow trout, Salmo gairdneri. Journal of Fish Biology 32, 63-76.

Graham, M.S., & Wood, C.M. (1981). Toxicity of environmental acid to the rainbow trout: interactions of water hardness, acid type, and exercise. Canadian Journal of Zoology 59, 1518-1526.

Graham, M.S., Wood, C.M., & Turner, J.D. (1982). The physiological response of the rainbow trout to strenuous exercise: interactions of water hardness and environmental acidity. Canadian Journal of Zoology 60, 3153-3164.

Heap, S.P., & Goldspink, G. (1986). Alterations to the swimming performance of carp, Cyprinus carpio, as a result of temperature acclimation. Journal of Fish Biology 29, 747-753.

Howells, G.D. (1984). Fishery decline: mechanisms and predictions. Philosophical Transactions of the Royal Society of London B305, 529-547.

Jensen, F.B. (1987). Influences of exercise-stress and adrenaline upon intra- and extracellular acid-base status, electrolyte composition and respiratory properties of blood in tench (Tinca tinca) at different seasons. Journal of Comparative Physiology B 157, 51-60.

Jones, D.R., & Randall, D.J. (1978). The respiratory and circulatory systems during exercise. In Fish Physiology, vol. 7 (Hoar, W.S. & Randall, D.J. eds), pp. 425-501. New York: Acadamic Press.

Lanctin, H.P., McMorran, L.G., & Driedzic, W.R. (1980). Rates of glucose and lactate oxidation by the perfused isolated trout (Salvelinus fontinalis) heart. Canadian Journal of

Zoology 58, 1708-1711.

Mazeaud, M.M., Mazeaud, F., & Donaldson, E.M. (1977). Primary and secondary effects of stress in fish: some new data with a general review. Trans. Mer. Fish. Soc. 106, 201-212.

McDonald, D.G. (1983a). The effects of H^+ upon the gills of freshwater fish. Canadian Journal of Zoology 61, 691-703.

Morata, P., Vargas, A.M., Pita, M.L., & Sanchez-Medina, F. (1982). Hormonal effects on the liver glucose metabolism in rainbow trout (Salmo gairdneri). Comparative Biochemistry and Physiology 72B, 543-545.

Ristori, M.T., & Laurent; P. (1985). Plasma catecholamines and glucose during moderate exercise in the trout: comparison with bursts of violent activity. Experimental Biology 44, 247-253.

Rome, L.C., Loughna, P.T., & Goldspink, G. (1984). Muscle fibre activity in carp as a function of swimming speed and muscle temperature. American Journal of Physiology 247, 272-279.

Salama, A., & Nikinmaa, M. (1988). The adrenergic responses of carp (Cyprinus carpio) red cells: effects of pO_2 and pH. Journal of Experimental Biology 136, 405-416.

Soivio, A., Westman, K., & Nyholm, K. (1972). Improved method of dorsal aorta catherization: haematological effects followed for three weeks in rainbow trout (Salmo gairdneri). Finnish fisheries research I, 11-21.

Soivio, A., Nyholm, K., & Westman, K. (1975). A technique for repeated sampling of blood of individual resting fish. Journal of Experimental Biology 63, 207-217.

Stevens, E.D., & Randall, D.J. (1967). Changes in gas concentrations in blood and water during moderate swimming activity in rainbow trout. Journal of Experimental Biology 46, 329-337.

Turner, J.D., Wood, C.M., & Clark, D. (1983). Lactate and proton dynamics in the rainbow trout (Salmo gairdneri). Journal of Experimental Biology 104, 247-268.

Ultsch, G.R., Ott, M.E., & Heisler, N. (1981). Acid-base and electrolyte status in carp (Cyprinus carpio) exposed to low environmental pH. Journal of Experimental Biology 93, 65-

80.

Van den Thillart, G., Randall, D., & Hoa-ren, L. (1983). CO_2 and H^+ excretion by swimming coho salmon, Oncorhynchus kisutch. Journal of Experimental Biology 107, 169-180.

Van den Thillart, G. (1986). Energy metabolism of swimming trout (Salmo gairdneri). Oxidation rates of palmitate, glucose, lactate, alanine, leucine, and glutamate. Journal of Comparative Physiology B 156, 511-520.

Van Dijk, P.L.M., & Wood, C.M. (1988). The effects of ß-adrenergic blockade on the recovery process after strenuous exercise in the rainbow trout, Salmo gairdneri Richardson. Journal of Fish Biology 32, 557-570.

Witters, H. (1990). Physiological study on the waterbug, Corixa punctata and the trout, Oncorhynchus mykiss: effects of aluminium in acid water. Ph.D. thesis, University of Antwerp, Belgium.

Wolf, K. (1963). Physiological salines for fresh-water teleosts. Progressive Fish-Culturist 25 135-140.

Wood, C.M. (1989). The physiological problems of fish in acid waters. In Acid Toxicity and Aquatic animals (ed. R. Morris, D.J.A. Brown, E.W. Taylor, & J.A. Brown), pp. 125-152. Cambridge: Cambridge University Press.

Wood, C.M., & Perry, S.F. (1985). Respiratory, circulatory, and metabolic adjustments to exercise in fish. In Circulation, Respiration and Metabolism. (ed. R. Gilles), pp. 2-22. Berlin: Springer.

Ye, X., & Randall, D.J. (1991). The effect of water pH on swimming performance in rainbow trout (Salmo gairdneri, Richardson). Fish Physiology and Biochemistry 9, 15-21.

CHAPTER 5:

THE INFLUENCE OF GRADUAL WATER ACIDIFICATION ON THE OXYGEN CONSUMPTION PATTERN OF FISH

ABSTRACT

Carp (Cyprinus carpio) and tilapia (Oreochromis mossambicus) were kept in a flow-through respirometer for a week. Continuous oxygen consumption measurements were carried out at a constant O_2 concentration. Whereas the control fish stayed in water of pH 7.6, for the other animals the water pH was gradually lowered to pH 4.0 and, for some fish, lateron temporarily to pH 3.2. For both carp and tilapia the average daily oxygen consumption in neutral water showed variation, but no consistent pattern could be discovered. We did not find any effect of on- or off-switching of the light on oxygen consumption, nor did we find any difference in oxygen uptake between daytime and nighttime for either species. In contrast to tilapia, carp reacted to water acidification with an extreme peak in O_2 uptake. It is discussed that tilapia and carp might have different strategies to survive acid episodes: whereas tilapia avoids the additional stress of exercise, carp tries to escape the acid conditions. Tilapia decreased its standard metabolic rate, its average, and its maximum oxygen consumption in acid water, whereas carp did not. Comparing our data with those from the literature, we conclude from these preliminary observations that the effect of acid exposure on carp is independent on whether the acidification procedure is gradual or acute. Our results indicate that both fish species become hypoxic in water of pH 3.2.

P. van Dijk, G. van den Thillart and S. Wendelaar Bonga; Comparative Physiology and Biochemistry, (accepted).

INTRODUCTION

Most laboratory studies on the effects of acid water on fish concern short-term exposures combined with acute acidification (see Wood, 1989, for review). The deleterious effects of acid water on fish, however, are much less when gradual, instead of acute water acidification is applied (see chapter 2, 3, 4 and 6).

The old idea of anoxia as the primary cause of death of fish in acid water (Westfall, 1945, Packer & Dunson, 1972) is no longer supported in the literature (Wood, 1989), though oxygen delivery problems do play a role in water of very low pH (<4.0; Ultsch et al., 1980; Wood & McDonald, 1982). In the present study with carp and tilapia, we were interested in the oxygen consumption pattern of these fish species, and how this pattern was affected by gradual water acidification. Is, after gradual water acidification, the oxygen consumption of fish decreased in water of pH 4.0, and how is the O_2 uptake affected at pH 3.2? In their study on carp, Ultsch et al. (1980) demonstrated that the standard metabolic rate and the aerobic scope for spontaneous exercise was not affected in the pH range of 7.4-4.0. At pH 3.5, however, both were decreased and at pH 3.0 an even further decrease was found. If we compare our data with those of Ultsch and co-workers, will we again come to the conclusion that the impact of water acidification on fish are much less after gradual acidfication (see chapter 2,3,4 and 6), or will we find that the effect on the oxygen uptake is more or less independent of the acidification procedure?

MATERIALS AND METHODS

Carp, Cyprinus carpio (300 ± 70 g, N=6), and tilapia, Oreochromis mossambicus (350 ± 40 g, N=5), of both sexes were obtained from local fish hatcheries and were held, for at least 6 months, in dechlorinated and well aerated local tap water of 20 ± 1°C for carp, and 25 ± 1°C for tilapia. The fish were kept

on a 14 L/10 D photocycle and fed daily with commercial fish food. At least two weeks before the start of the experiments the fish were transferred to four times diluted local tap water (1 vol of copper-free tap water mixed with 3 vols of demineralized water; end values: Na^+ 0.83, Cl^- 0.92, Ca^{2+} 0.68, K^+ 0.07, Mg^{2+} 0.16, NO_3^-0.03, HCO_3^-1.11, SO_4^{2-}0.29, SiO_2 0.06 mmol l^{-1}; $Al_{tot.}$ was below the detection level of 6.7 nmol l^{-1}). During the acclimation period and throughout the experiments the fish were kept in this water at 20.0 ± 0.5°C for carp, and 25.0 ± 0.5°C for tilapia. Feeding was suspended at least 24 hours before experimentation. Experiments with tilapia were performed in March and April, with carp in the period April - June.

On day one the fish was placed in the 12.3 l respirometer as described by Van den Thillart and Verbeek (1991). The fish stayed in this set-up for one week. The oxygen tension inside the respirometer was kept at a set point (60 - 70% air-saturated water). Whenever the oxygen level decreased below this point, air-saturated water was let into the respirometer, until the set point was reached again. The oxygen consumption was measured continuously over 15-minutes intervals by means of automatic flowmeter readings (see Van den Thillart & Verbeek, 1991, for details concerning oxygen consumption measurements). The following equation was used to calculate the metabolic rate (= oxygen consumption) for a 100g fish:

$$M_{100} = M_w \cdot [100 / W]^{-0.8}$$

where M_w is the measured metabolic rate, and W is the mass of the fish. Oxygen uptake measurements have been corrected for drift of the O_2 electrode. To this end, Winkler oxygen titrations of the in- and outflowing water were performed on a daily basis. Blank oxygen consumption was found to be less than 2% of the total O_2 uptake and was neglected.

For the control fish a pH-stat device kept the water at 7.6 with 1.0 mmol l^{-1} NaOH. For the other fish, on day five, we gradually lowered the water pH to pH 4.0, using a pH-stat device (METROHM 605/614/655) and 0.5 mmol l^{-1} H_2SO_4. This procedure took about five hours. For some fish the water pH was further decreased on day 7. In one hour the water pH was gradually

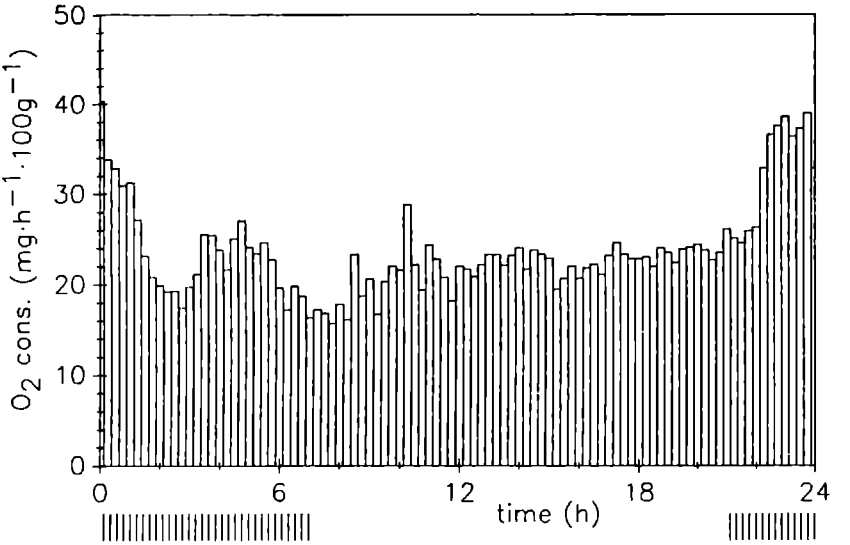

Fig. 1: An example of the oxygen consumption pattern over one day of a carp kept in neutral (pH 7.6) water. These measurements were taken over 15-minutes intervals, on day 7. Vertical stripes indicate period of darkness.

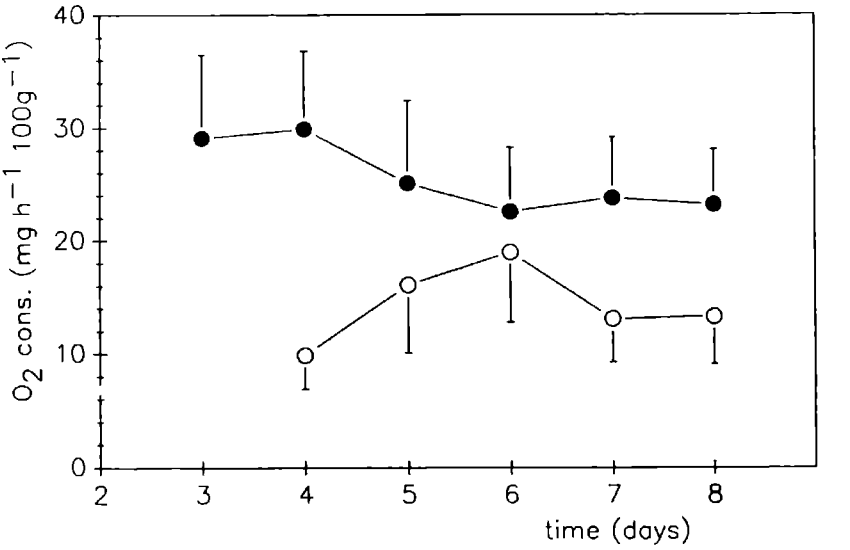

Fig. 2: The average daily (over 24 hours) oxygen consumption (ADOC) of two carp kept in water of pH 7.6 for a week. Mean ± SD.

lowered to 3.2, the pH was kept at this value for four hours,before the water pH was brought back to pH 4.0 in one hour. The fish remained at this pH until day eight, when the water pH was again increased to pH 7.6. After at least one hour in neutral water the fish was removed from the respirometer and the animal's body mass was determined. A pH-stat device kept the water pH at the desired value troughout the experiment. The pH electrodes (Russell CTL/LCW for low conductivity) that were employed, were specially designed to be used in solutions of low ionic strength. All values are expressed as means ± SD. Presented P values are based on the Mann-Whitney U-test.

RESULTS

One of the two carp (carp #2, Table 1) that was exposed to water of pH 3.2 died in the night following the end of the experiment. At this time the fish was kept in neutral water. There were no additional mortalities among carp or tilapia during or after the experiment.

Fig. 1 shows a representative example of the oxygen consumption pattern of a carp kept in water of pH 7.6. These measurements were taken on day 7. As can be seen the oxygen uptake is not constant: periods of relative constant oxygen consumption alternate with periods of various length of increased oxygen uptake. The average daily oxygen consumption (ADOC) of carp in neutral water showed variation but no consistent pattern could be discovered (Fig. 2). The ratio of the largest to the smallest ADOC measured over the period day 3 (day 4) - day 8 for the two carp kept in neutral water was 1 : 0.76 and 1 : 0.52, respectively. In Table 1 the minimum, the maximum, and the average oxygen consumption of carp over periods of four hours are shown. It can be seen, that carp usually reacted on water acidification with an extreme peak in oxygen uptake. In three out of four fish that were exposed to water of pH 4.0, we observed a significant decrease in maximum oxygen uptake.

	pH7.6	pH ↓	pH 4.0	pH 3.2	pH 4.0′
carp #1					
mean	11.0 ±2.0	14.2 ±3.8	9.65 ±.94	9.9 ±2.0	14.57 ♦♦ ±.92
max.	16.3 ±4.9	25.8	11.8 * ±2.0	15.3	16.50 ♦ ±.44
min.	7.45 ±.53	9.7	7.79 ±.34	7.8	12.67 ♦♦ ±.85
carp #2					
mean	25.1 ±4.8	43.0 ±8.0	25.6 ±2.4	14.1 ±3.7	10.3 ♦♦ ±2.2
max.	30.2 ±5.2	55.8	32.0 ±4.5	23.8	12.3 ♦♦ ±1.1
min.	20.4 ±4.9	29.2	21.9 ±2.2	11.0	8.1 ♦♦ ±2.1
carp #3					
mean	16.2 ±5.5	33.0 ±13.0	11.0 ±4.8		
max.	23.1 ±6.3	48.0	15.6 * ±8.7		
min.	9.1 ±4.3	6.5	7.2 ±3.0		
carp #4					
mean	10.6 ±1.5	11.6 ±2.0	7.95 ** ±.80		
max.	12.7 ±2.1	18.7	9.44 ** ±.97		
min.	8.9 ±1.3	10.0	6.3 ** ±1.1		
	pH 7.6		pH 7.6′		
carp #5					
mean	28.7 ±5.7		23.9 ±5.1		
max.	37.1 ±7.5		30.1 * ±7.9		
min.	21.1 ±5.6		18.9 ±3.3		
carp #6					
mean	11.8 ±5.0		15.0 ±5.5		
max.	16.8 ±5.7		18.7 ±6.4		
min.	7.7 ±2.8		11.7 ±5.2		

Table 1: The mean, maximum (max.), and minimum (min.) oxygen consumption (in mg h^{-1} $100g^{-1}$) of carp, taken over four hour periods. Mean ± SD.

pH 7.6 : measurements while the fish was in neutral water;
pH ↓ : measurements during four hours of the water acidification period;
pH 4.0 : measurements while the fish was in water of pH 4.0;
pH 3.2 : measurements during the four hours at pH 3.2;
pH 4.0' : measurements in water of pH 4.0, after the fish returned from water of pH 3.2;
pH 7.6' : measurements in neutral water in an identical period as for the fish that were exposed to water of pH 4.0;

* significant difference between the measurements at pH 7.6 and the first pH 4.0 period;
♦ significant difference between the two pH 4.0 periods;
* or ♦: P≤0.05; ** or ♦♦: P≤0.01.

Comparing this parameter over identical periods (in Table 1 under pH 7.6 and pH 7.6') with control fish gave a similar result, so this decrease is probably not due to water acidification.

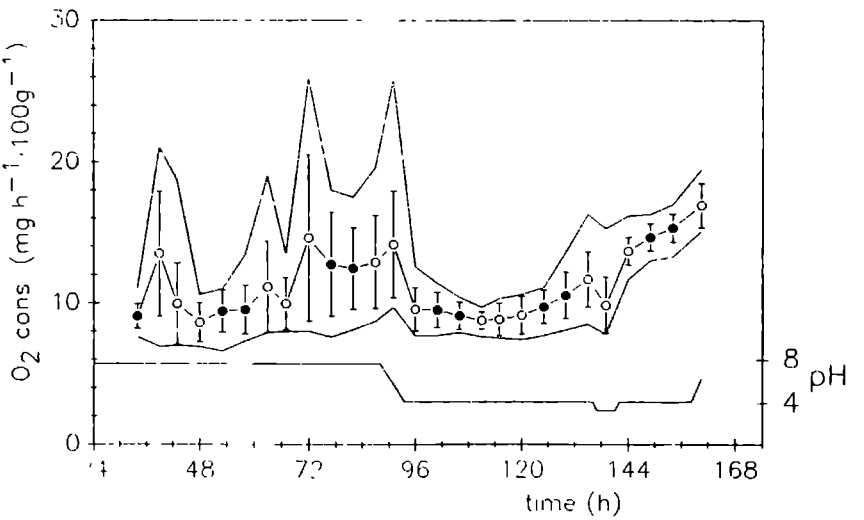

Fig. 3: The minimum (standard metabolic rate; SMR), the maximum, and the average oxygen consumption (± SD) over periods of four hours of carp #1. Open circle: measurements during the light period; filled circles: measurements during the dark period. The lowest line shows the water pH.

Only in carp #4 we found a decrease in mean and minimum oxygen consumption at pH 4.0, in the other fish these values were similar in acid and in neutral water. This is also shown in Fig. 3, which is a more detailed graph of the influence of the water pH on the minimum, the maximum and the average oxygen consumption (over four hour periods, for carp #1). At pH 3.2 these three parameters remained at a similar level as before, but after this low pH period we measured a gradual increase in the minimum, and the average oxygen uptake of this carp.

In Fig. 4 an example of the daily oxygen consumption pattern of a tilapia kept in neutral water (pH 7.6) is shown. On this day, for this fish, the oxygen consumption was fairly constant, without any peaks, however, on other days this fish also had peaks in oxygen uptake, but similar as in carp there was no relationship with the switching of the light.

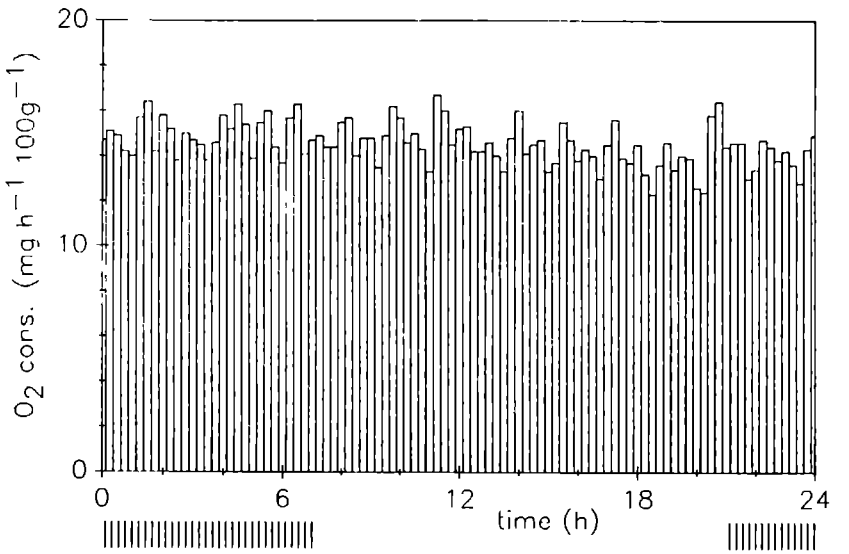

Fig. 4: An example of the oxygen consumption pattern, over a day, of a tilapia kept in neutral (pH 7.6) water. These measurements were taken over 15-minutes intervals, on day 6. Vertical stripes indicate period of darkness.

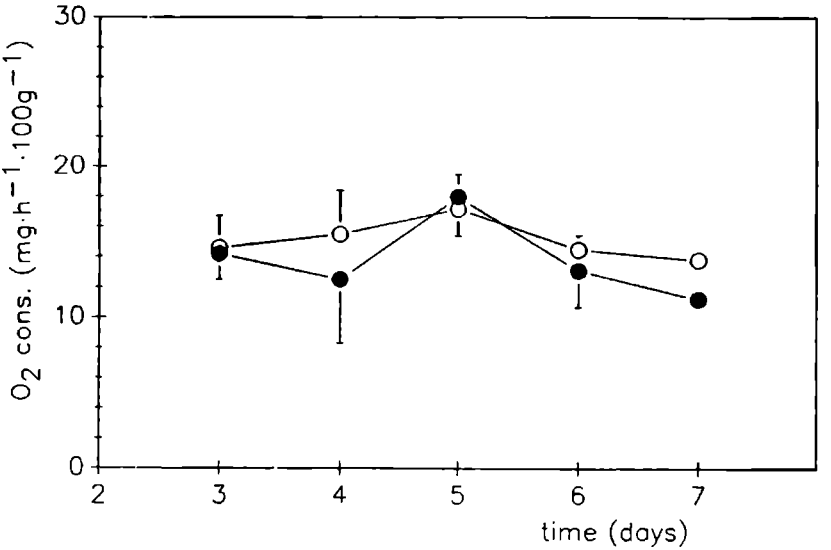

Fig. 5: The average daily (over 24 hours) oxygen consumption (ADOC) of two tilapias kept in pH 7.6 water for a week. Mean ± SD.

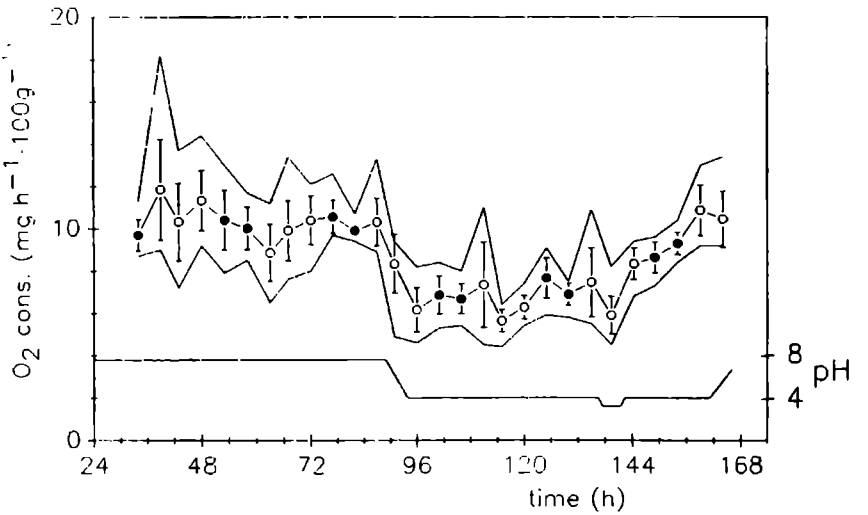

Fig. 6: The minimum (standard metabolic rate; SMR), the maximum, and the average oxygen consumption (± SD) over periods of four hours of tilapia #1. Open circle: measurements during the light period; filled circle: measurements during the dark period. The lowest line shows the water pH.

	pH 7.6	pH ↓	pH 4.0	pH 3.2	pH 4.0′
tilapia #1					
mean	10.30 ±.76	8.3 ±1.4	6.77 ** ±.66	5.90 ±.90	8.76 ◆◆ ±.50
max.	13.0 ±2.0	9.4	8.5 ** ±1.6	8.2	9.80 ◆ ±.53
min.	8.38 ±.96	4.9	5.20 ** ±.56	4.5	7.50 ◆◆ ±.82
tilapia #2					
mean	19.7 ±4.0	19.7 ±2.3	11.2 ** ±1.8		
max.	23.5 ±5.2	23.8	13.6 ** ±2.9		
min.	15.1 ±3.0	15.2	9.2 ** ±1.1		
tilapia #3					
mean	18.0 ±2.2	16.3 ±2.0	11.1 ** ±1.1		
max.	20.7 ±2.1	19.1	12.6 ** ±2.1		
min.	16.5 ±1.7	11.9	10.1 ** ±1.0		

	pH 7.6	pH 7.6′
tilapia #4		
mean	15.4 ±1.9	14.7 ±1.1
max.	17.8 ±2.7	16.3 ±1.4
min.	12.6 ±2.1	13.2 ±1.0
tilapia #5		
mean	13.5 ±3.4	13.5 ±2.9
max.	15.4 ±3.6	15.0 ±3.5
min.	12.0 ±3.4	12.2 ±2.7

Table 2: The mean, maximum (max.), and minimum (min.) oxygen consumption (in mg h^{-1} $100g^{-1}$) of tilapia, taken over four hour periods. Mean ± SD. Table legend as for Table 1.

In addition, no difference was found, for both fish species, between daytime and nighttime oxygen uptake. Furthermore, we did not find, for tilapia or carp, that the number of peaks per day decreased over the period day 3 - day 8.

The ADOC for tilapia kept in water of pH 7.6 showed some variation (Fig. 5). The ratio of the largest to the smallest ADOC measured over the period day 3 - day 7 for these two tilapia was 1 : 0.80 and 1 : 0.62. Fig. 6 shows an example of the minimum, maximum, and average oxygen consumption over periods of four hours of tilapia during the experiment. All three parameters showed a clear decrease in level after lowering the water pH from 7.6 to 4.0 (see also Table 2).

No such change occurred in the control animals (Table 2). A further decrease of the water pH to 3.2, however, did not cause any additional change in these three parameters, but all three showed a gradual increase after alkalinization to pH 4.0 (Fig. 6). With tilapia, we never observed a peak in oxygen consumption as a reaction to an acidification procedure (Table 2).

DISCUSSION

In the present study the environmental oxygen tension inside the respirometer was kept at 60 - 70% air-saturation. Hughes *et al.* (1983) found with carp at 20°C a similar respiration rate at 60%, as at 100% air-saturation. With rainbow trout at 15°C, Bushnell *et al.* (1984) could even go down to 27% air-saturation, without finding a significant change in oxygen uptake. It can therefore be expected that our oxygen uptake measurements were not affected by the very mild hypoxia inside the respirometer.

The minimum oxygen consumption rates that we measured during the four hour periods, could be taken as a measure for the standard metabolic rate (SMR) for this four hour period. In their study on carp Ultsch et al. (1980) report that "the standard metabolic rate was taken at the average oxygen uptake during the first hour of low activity when the calculated oxygen consumption did not vary by more than 20%". Ultsch and co-workers allowed the fish to habituate and to recover from the handling stress for at least 8 hours before the start of the experiment. In the present study, carp were allowed to recover for at least 30 hours before our first measurements. We observed that tilapia during the pH 3.2 period became completely motionless, but at other times it can not be excluded that even during the 15 minutes of minimum oxygen uptake the fish performed some activity. Still our minimum oxygen uptake measurements were in the range of values known from literature for SMR, or oxygen uptake of resting fish (Saunders, 1962; Ultsch et al., 1980; Takeda, 1989; chapter 6 for data on carp; Caulton, 1978; Mishrigi & Kubo, 1978; Ross & Ross, 1983; Ross & McKinney, 1988 for data on tilapia). In conclusion, we feel confident to call the minimum value, over a four hour period, the SMR of the fish for that period.

The most important result of the present study is the drastic decrease of SMR and of maximum metabolic rate, and average oxygen consumption level in tilapia after gradual acidification of the water to pH 4.0. From our measurements it can be calculated that the decrease in SMR is responsible for more than 80% of the reduction in average oxygen consumption, and for about 70% of the decrease in maximum oxygen uptake. A reduction of spontaneous activity, both in duration and intensity, but not in frequency, was probably responsible for the remaining part. With carp the minimum oxygen consumption level was, except for one fish, not affected by water acidification, so we think that carp does not decrease its standard metabolic rate in acid water. This is in agreement with another study on the effect of acid exposure on carp (Ultsch et al., 1980). In the present study we used gradual water

acifification, whereas Ultsch and co-workers used an instant acidification procedure. Nevertheless, both studies gave a similar result, so we conclude that unlike other parameters such as plasma sodium, chloride, glucose, and arterial pH (see chapter 3,4 and 6) the effect of acid exposure on the oxygen consumption of carp is independent on whether the acidification procedure is gradual or instant. So the gill damage that, as Wendelaar Bonga *et al.* (1987) demonstrated, only takes place after an instant procedure, seems not to affect the oxygen uptake of carp in acid water (pH 4.0). The reduction in the maximum, that we found in three acid exposed fish and in one control fish, must have been caused by the reduction of the spontaneous activity of these carp in the last part of the experiment.

In this study we found that the oxygen consumption of carp and tilapia is variable with time. This was also found by many other authors in several fish species, including carp (Huang, 1975; Hamada and Maeda, 1983), and tilapia (Nagarajan and Gopal, 1983). For both fish species, we did not observe differences in level between our daytime and nighttime oxygen uptake measurements. This is in contrast with the study from Ross and McKinney (1988) on Nile tilapia (*Oreochromis niloticus*), who found a higher respiratory rate during the light period than during darkness.

The two fish species used in the present study reacted differently on the onset of water acidification. Tilapia became motionless, whereas, carp usually reacted with intense movements, causing a peak in oxygen uptake. A similar, but even stronger reaction was observed by Ultsch *et al.* (1980), who reported that both rainbow trout and carp reacted on the addition of acid to the water with struggling, sometimes violently. From field observations it is known that acid episodes cause changes in behaviour patterns of fish. There is evidence that fish show avoidance reactions to low pH conditions, and it is possible that during episodes, particularly in large water bodies, they could locate areas of more favourable conditions, e.g. deeper water or upwelling

ground water (Gunn, 1986). It was observed that salmon spawners moved downstream, the reverse of their expected behaviour pattern, away from the source of the acid aluminium-rich water (Skogheim et al., 1984). We suggest that carp also tried to escape the acid water in our experiment. Tilapia, however, seems to have a different strategy to survive acid conditions. In our earlier experiments we found that moderate exercise is an additional stressor in acid water (see chapter 3 and 4). So obviously, tilapia avoids this additional stress by becoming motionless. We have some evidence for this fish species that animals exposed to acid water (3 days at pH 4.0) have a lower turnover rate of catecholamines than control fish (P. Balm, P. Van Dijk, G. Van den Thillart, and S. Wendelaar Bonga, unpublished results). So, this lower turnover rate could be responsible for the observed behaviour of tilapia.

In the tilapia and in one of the two carp, we saw a rise in minimum oxygen uptake level, as we increased the water pH from 3.2 to 4.0. Ultsch et al. (1980) concluded that carp becomes hypoxic in pH 3.5 water. Most likely our fish also became hypoxic during the four hours at pH 3.2. The increase in oxygen consumption in the following period would then represent the compensation of the oxygen debt. The one carp (#2) that did not increase its oxygen uptake after the pH 3.2 period, died in the night following the experiment. After the period of extreme low water pH this fish was lying on its side on the bottom of the respirometer. So we think that this carp also became hypoxic, but unlike the other fish was not able to recover.

Bushnell, P.G., Steffensen, J.F., & Johansen, K. (1984). Oxygen consumption and swimming performance in hypoxia-acclimated rainbow trout Salmo gairdneri. J. exp. Biol. 113, 225-235.

Caulton, M.S. (1978). The effect of temperature on routine metabolism in Tilapia rendali Boulenger. J. Fish Biol. 11, 549-553.

Gunn, J.M. (1986). Behaviour and ecology of salmonid fishes exposed to episodic pH depressions. Envir. Biol. Fish. 17, 241-252.

Hamada, A., & Maeda, W. (1983). Oxygen uptake due to specific dynamic action of the carp Cyprinus carpio. Jap. J. Limnol. 44, 225-239.

Huang, Y.Y. (1975). Oxygen consumption of the early development stages of the red and the common carp (Cyprinus carpio), the silver carp (Hypopthalmichthys molitrix) and the chinese bream (Parabramis pekinensis). Acta. Zool. Sin. 21, 78-88.

Hughes, G.M., Albers, C., Muster, D., & Götz, K.H. (1983). Respiration of the carp, Cyprinus carpio L., at 10 and 20 °C and the effects of hypoxia. J. Fish Biol. 22, 613-628.

Mishrigi, S.Y., & Kubo, T. (1978). The energy metabolism in Tilapia nilotica. II. Active metabolism at 10 and 20°C. Bull. Fac. Fish. Hokaido. Univ. 29, 100-109.

Nagarajan,K., & Gopal, V. (1983). Effect of photoperiod on oxygen consumption and food utilization in Tilapia mossambica Peters. Proc. natn. Acad. Sci. India. 53B, 217-225.

Packer, R.K., & Dunson, W.A. (1972). Anoxia and sodium loss associated with the death of brook trout at low pH. Comp. Biochem. Physiol. 41A, 17-26.

Ross, L.G., & McKinney, R.W. (1988). Respiratory cycles in Oreochromis niloticus (L.), measured using a six-channel microcomputer-operated respirometer. Comp. Biochem. Physiol. 89A, 637-643.

Ross, B., & Ross, L.G. (1983). The oxygen requirements of

Oreochromis niloticus under adverse conditions. In Proceedings of the first International Symposium on Tilapia in Aquaculture, Nazareth, Israel, 8-13 May 1983. (Compiled by Fishelson L., & Yaron, Z.) pp. 134-143. Tel-Aviv University Press, Tel Aviv.

Saunders, R.L. (1962). The irrigation of the gills in fishes. II. The efficiency of oxygen uptake in relation to respiratory flow, activity and concentrations of oxygen and carbon dioxide. Can. J. Zool. 40, 817-862.

Skogheim, O.K., Rosseland, B.O. and Sevaldrud, I. (1984). Deaths of spawners of Atlantic salmon in River Ogna, S.W. Norway, caused by acidified aluminium-rich water. Rep. Inst. Freshwater Res. Drottningholm 61, 195-202.

Takeda, T. (1989). Cutaneous and gill O_2 uptake in the carp, Cyprinus carpio, as a function of ambient p_{O2}. Comp. Biochem. Physiol. 94A, 205-208

Ultsch, G.R., Ott, M.E., & Heisler, N. (1980). Standard metabolic rate, critical oxygen tension, and aerobic scope for spontaneous activity of trout (Salmo gairdneri) and carp (Cyprinus carpio) in acidified water. Comp. Biochem. and Physiol. 67A, 329-335.

Van den Thillart, G., & Verbeek, R. (1991). Anoxia-induced Oxygen Debt of Goldfish (Carassius auratus L.). Physiol. Zool. 64(2), 525-540.

Wendelaar Bonga, S.E., Flik, G. and Balm, P.H.M. (1987). Physiological adaptation to acid stress in fish. Ann. Soc. R. Zool. Belg. 117, 243-254.

Westfall, B.A. (1945). Coagulation film anoxia in fishes. Ecology 26, 283-287.

Wood, C.M. (1989). The physiological problems of fish in acid waters. In Acid Toxicity and Aquatic animals (ed. R. Morris, E.W. Taylor, D.J.A. Brown, & J.A. Brown), pp. 125-152. Cambridge: Cambridge University Press.

Wood, C.M., & McDonald, D.G. (1982). Physiological mechanism of acid toxicity to fish. In Acid Rain/Fisheries (ed. R.E. Johnson), pp. 197-226. Bethesda: American Fisheries Society.

CHAPTER 6

EFFECTS OF LONG-TERM EXPOSURE TO ACID WATER ON OXYGEN CONSUMPTION AND CRITICAL SWIMMING SPEED OF CARP

ABSTRACT

Juvenile carp were allowed to acclimate for at least four weeks to water of pH 5.0 or 7.6, before they were subjected to the following procedure: on day 1 a carp was transferred into a Blazka swim tunnel respirometer. After a training period, the critical swimming speed was determined on day 6. The next day the carp swam twice for half an hour at 80% of the critical velocity. On day 8, immediately after 15 minutes of exhaustive exercise, terminal blood samples were taken. Water of pH 5.0 was rather well tolerated by carp. No difference occurred in critical swimming speed between the pH 7.6 and the pH 5.0 group. Four weeks of exposure to pH 5.0 obviously did not affect the ability of carp to perform physical exercise. At swimming speeds between 1.5 and 3.5 BL s^{-1} there was no difference in oxygen consumption between the two groups. During strenuous exercise, however, the acid exposed fish consumed less oxygen than the control animals, whereas the plasma lactate levels were not different between the two groups. This could be an indication that at velocities above critical speed carp swam with a higher efficiency at pH 5.0 than at pH 7.6. The occurrance of an enhanced haematocrit and a lower plasma chloride concentration in the pH 5.0 group after strenuous exercise were the only indications that prolonged exposure to low pH had negative effects on carp. These results substantiate our conclusion from earlier studies (see chapters 3 and 4) that the effect of acid water on fish has often been overestimated in literature. Application of short-term exposure periodes or additional stress, such as handling or an instant acidification procedure, probably caused this overestimation.

P. van Dijk, G. van den Thillart and S. Wendelaar Bonga; Physiological Zoology (submitted).

The physiological effects of acid water on fish have received ample attention in recent years (see Wood 1989, for review). There are also some studies on the combined effects of exercise and acid water (Waiwood and Beamish 1978; Graham and Wood 1981; Graham, Wood, and Turner 1982; Nelson 1989; Nelson 1990; Ye and Randall 1991; Ye, Randall, and He 1991; Butler, Day, and Namba 1992; Nelson and Mitchell 1992; and see chapter 4). With rainbow trout, Waiwood and Beamish (1978) did not find any effect of exposure to pH 6.0 on critical performance. Lower pH values, however, led to an significantly decreased swimming speed in this species (Ye and Randall 1991). Similar results were obtained with brown trout at pH 4.5 (Butler et al. 1992) and tilapia Oreochromis mossambicus at pH 4.0 (P. Van Dijk, G. Van den Thillart and S. Wendelaar Bonga, unpublished results). Similarly, yellow perch adapted to pH 4.4 had a lower critical swimming speed in water of pH 4.0 than fish adapted to and tested in neutral water (Nelson 1989). In carp, gradual water acidification in combination with moderate exercise caused ionoregulatory failure, hyperglycemia, and a constant declining blood pH, phenomena that did not occur in carp at rest (see chapters 3 and 4).

In most of these studies the fish were kept before experimentation in water of a higher pH than during the experiments. We wanted to investigate if the critical swimming speed was also affected after pre-exposure of the fish to the experimental pH. Prolonged exposure to acid water does not lead to complete physiological adaptation of fish, although long-term exposed animals do reach a new physiological steady state (Wendelaar Bonga et al. 1987; Audet, Munger, and Wood 1988; Audet and Wood 1988), and this may enhance the physiological performance. McWilliams (1980) found that brown trout needed about two weeks to acclimate to pH 6.0. According to him, the acclimation to acid conditions seems mainly dependend on changes in gill permeability. We examined two groups of carp that were allowed to acclimate for at least four weeks to the same pH as

during the experiment (pH 7.6 and pH 5.0, respectively). Environmentally realistic pH levels were used, as in nature carp populations are rare in water of below pH 5.0 (Leuven et al. 1987). In addition, we investigated the relation between oxygen consumption and swimming speed for both groups. The results of this study are environmentally relevant because a possible reduced physical performance will have a negative impact on the survival prospects of fish populations.

MATERIALS AND METHODS

Juvenile carp, Cyprinus carpio, (body mass 13.9 ± 1.1 g; length 7.64 ± 0.19 cm)) of both sexes were obtained from the Experimental Fish Culture Station, Agricultural University of Wageningen, The Netherlands, and were held for at least one month at 20.0 ± 0.5°C in well aerated freshwater containing: Na^+ 0.83, Cl^- 0.92, Ca^{2+} 0.68, K^+ 0.07, Mg^{2+} 0.16, NO_3^- 0.03, HCO_3^- 1.11, SO_4^{2-} 0.29, SiO_2 0.06 mmol l^{-1}; $Al_{tot.}$ was below the detection level of 6.7 nmol l^{-1}. A pH-stat device, titrating 1 mol l^{-1} NaOH, was used to maintain the water pH at 7.6. The fish were kept on a 14 L/10 D photocycle and fed daily with cichlid food in flake form (Lapis, Europet, Nürnberg, FRG).

Two groups of fish were used: a control group (N=7), for which the water pH was kept at 7.6, and a pH 5.0 group (N=6), for which the water pH was gradually lowered (from pH 7.6 to pH 5.0 in 4 hours) and then kept at this pH with a pH-stat device (METROHM 605/614/655) titrating 0.5 mol l^{-1} H_2SO_4. Fish were pre-exposed to these conditions for 4 to 6 weeks before experimentation. The experiments were performed from February to April.

On day 0, every experimental fish was fed for the last time. On day 1, its length, maximal body height, maximal body width, and mass were determined, after which the fish was put into the Blazka swim tunnel respirometer (Blazka, Volf, and Cepela 1960) The swim tunnel was the same as described by Smit et al. (1971) with sizes reduced to 35 cm length and 10 cm

diameter. The respirometer was filled with water of the same composition, temperature, and pH, as during the pre-exposure period. During day 2 and 3 the fish was allowed to acclimate to the new surroundings and to recover from the handling stress. During these days and during all the nights (17.30h-8.30h), the swim tunnel was not running. On day 4 and 5 the fish was trained intensely to swim at the whole range of swimming speeds, without exhausting the fish. A small lightbulb mounted under the anterior grid of the swim tunnel served as visual orientation point for the fish. To prevent the carp from resting during the exercise protocol, a grid made of silver wire, carrying a 3 V alternating current, was placed behind the fish. On day 6 we first measured the oxygen consumption of resting and undisturbed carp. To this end the water supply to the respirometer was temporarily shut off and closed with rubber stoppers. The rate at which the oxygen concentration inside the swim tunnel decreased was used to calculate the oxygen consumption of the fish. The following equation was used to calculate the metabolic rate (= oxygen consumption) for a 100g fish:

$$M_{100} = M_w \cdot [100 / W]^{-0.8}$$

where M_w is the measured metabolic rate, and W is the mass of the carp. The O_2 concentration inside the respirometer was measured with an EIL oxygen measuring system (Electronic Instruments Ltd., Kent) and recorded constantly. Winkler oxygen titrations were performed once a day to check and calibrate the oxygen electrode. After this first oxygen consumption measurement, we started the water flow inside the swim tunnel and forced the fish to swim at 1 body length per second (1 BL s^{-1}) for half an hour. During this period we measured the average oxygen uptake of the fish. In the calculation of the swimming speed of the fish, we made a correction for the local acceleration of the water flow, caused by the narrowing of the streambed by the animal's body, under the assumption that the cross-sectional area of the fish is an ellipse (Smit et al. 1971). To determine the critical velocity of the carp, the swimming speed was increased with 0.5 BL s^{-1} increments every 0.5 hour interval, until the fish were exhausted (see Beamish

1978, for details concerning calculation of critical velocity). At each speed level the O_2 consumption was measured. During these measurements the oxygen concentration within the respirometer did not decrease below 80-85% air-saturation. The next day we again determined the oxygen uptake of the carp in the resting and undisturbed state. Then the swim tunnel was started and the speed of the fish was slowly increased until, within 10 minutes, a velocity equal to 80% of the critical velocity was reached. While the carp swam at this speed for half an hour, we measured the average O_2 uptake of the fish. Then the swim tunnel was switched off and the oxygen consumption during the first 15-20 minutes of this resting state was determined. At least two hours later, the swim tunnel was turned on again and this exercise and rest procedure was repeated. On the following day (day 8) the oxygen consumption was determined while the fish was forced to swim at maximal speed for 15 minutes. During this period the swimming speed never exceeded 120% of the critical velocity. After this period of exhaustive exercise the fish was quickly removed from the respirometer and killed with a blow on the head. A terminal blood sample was taken by cutting off the tail and collecting the blood in heparinized tubes. This whole procedure did not take more than 2 minutes.

The hemoglobin concentration and the haematocrit of the whole blood were determined as well as the concentrations of sodium, chloride, potassium, lactate, glucose, and cortisol of the blood plasma. The analytical methods used were described in chapter 3.

Data were expressed as means ± S.E.M. Data were tested for significance with Student's two-tailed t-test, except for the oxygen uptake, which was tested with Mann-Whitney U-test. Statistical significance was accepted at $P \leq 0.05$.

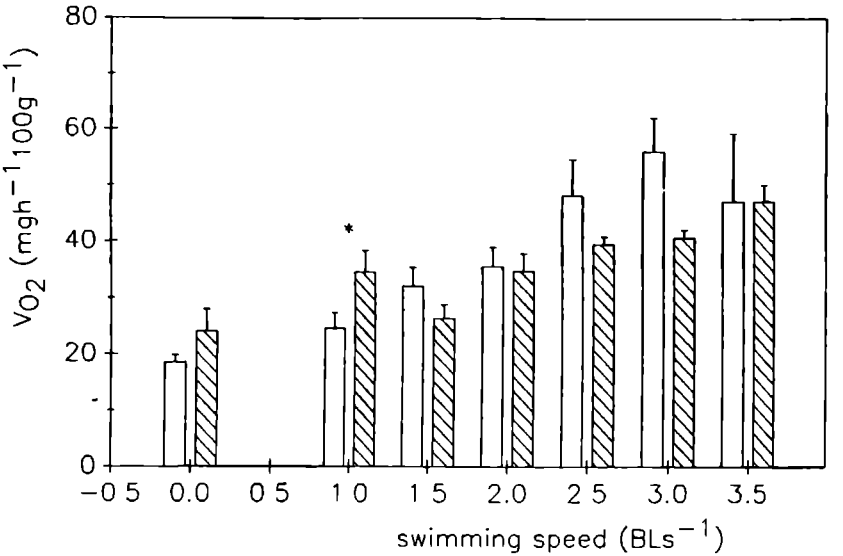

Fig. 1: Oxygen consumption of juvenile carp at various speeds. Mean ± S.E.M. Blank box: control group, fish were adapted to, and exercised in pH 7.6 water; hatched box: pH 5.0 group, carp previously held (for four to six weeks) in pH 5.0 water; *: significant difference (P≤0.05) between the two groups.

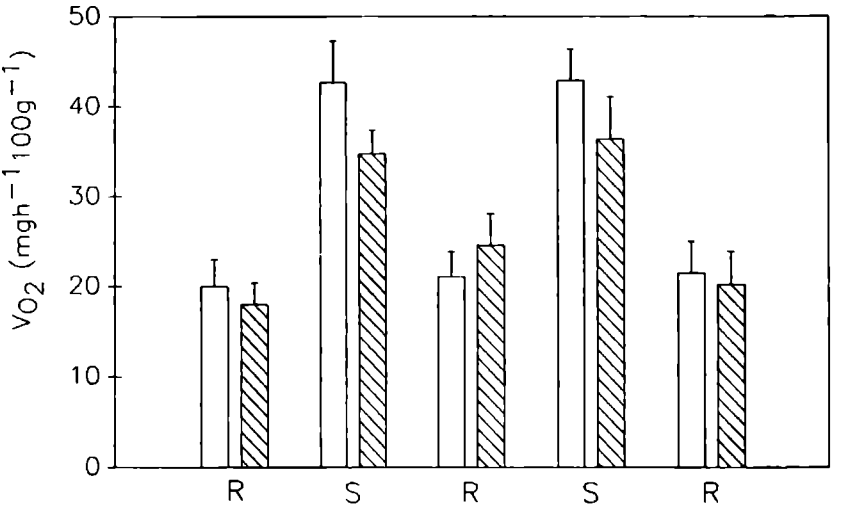

Fig. 2: Oxygen consumption of juvenile carp. Mean ± S.E.M. R: period of resting; S, period of half an hour of swimming at 80% of the critical speed. Other details as in fig. 1.

All fish survived until the end of the experimental period. We found no significant difference in critical swimming speed between the pH 7.6 group (2.91 ± 0.16 BL s^{-1}; N=7) and the pH 5.0 group (3.24 ± 0.32 BL s^{-1}; N=6). The oxygen uptake did not differ between the two groups, neither at rest, nor while constantly swimming at 1.5 - 3.5 BL s^{-1} (figure 1). Only at a swimming speed of 1.0 BL s^{-1}, the oxygen consumption of the pH 5.0 group was higher than that of the control group.

Figure 2 shows the oxygen consumption rates of the two experimental groups during alternating periods of rest and swimming at 80% of the critical velocity. There were no significant differences between the two groups with respect to the O_2 consumption neither at rest nor during swimming. Within each group the respiratory rate during the first and the second swimming period were at the same level. Furthermore, the oxygen consumption rates that were measured during the 3 resting periods preceding and following the 2 swimming periods were similar in both groups.

	control		pH 5.0
haematocrit (%)	23.2 ± 0.9	*	27.7 ± 1.6
MCHC (g l^{-1})	264 ± 29		260 ± 12
Na^+ (mmol l^{-1})	160.4 ± 5.8		147.6 ± 5.7
Cl^- (mmol l^{-1})	152.6 ± 4.1	*	134.8 ± 4.8
K^+ (mmol l^{-1})	12.1 ± 1.0		13.1 ± 1.5
lactate (mmol l^{-1})	7.7 ± 2.6		4.1 ± 1.0
glucose (mmol l^{-1})	1.04 ± .13		1.27 ± .09
cortisol (µmol l^{-1})	.84 ± .11		1.02 ± .47

Table 1: Blood parameters in juvenile carp. The blood samples were taken immediately after 15 minutes of strenuous exercise. Except for the haematocrit value and the mean cell hemoglobin concentration (MCHC), which were measured in whole blood, all parameters were measured in plasma. Mean ± S.E.M.
*: significant difference ($P \leq 0.05$) between the two groups.

The oxygen consumption, measured during the 15 minutes of strenuous exercise on day 8, was significantly lower for the pH 5.0 group (45.5 ± 5.1 mg h^{-1} $100g^{-1}$) than for the control group (71.6 ± 9.8 mg h^{-1} $100g^{-1}$). In the blood samples taken

immediately after this period of exhaustive exercise, no significant difference was found between the two experimental groups with respect to mean cell hemoglobin concentration, and plasma sodium, potassium, lactate, glucose, and cortisol concentration (Table 1). A higher haematocrit and a lower plasma chloride concentration was observed in the pH 5.0 group.

DISCUSSION

Our most remarkable result is that we did not find any difference in critical velocity between carp swimming in pH 7.6, and in pH 5.0 water. This is in agreement with the study of Waiwood and Beamish (1978) who tested critical performance of rainbow trout at pH 6.0. In the same species, Ye et al. (1991) found a reduction in critical velocity at pH 5.0. Our fish were held in the acid water for at least 4 weeks before the experiment started, while in the study of Ye and co-workers the fish were transferred from neutral water to water of pH 5.0 shortly before the experiment. We suggest that this difference in experimental procedure between both studies caused the difference in result. Nelson (1989) found that yellow perch pre-exposed to pH 4.4 had a lower critical swimming speed in water of pH 4.0 than fish adapted to and tested in neutral water. This may seem at variance with our results. However, there are a few differences in protocol between the two studies. Firstly, our experiments with juvenile carp were carried out at pH 5.0 while Nelson exposed perch (mostly gravid females) to pH 4.0. Secondly, in contrast to our experiment, the test pH in Nelson's study was lower than the pre-exposure pH. As was shown by Audet and Wood (1988), long-term sublethal acid exposure decreases the ability of rainbow trout to respond to more severe acid conditions. So the lower critical speed of the pre-exposed fish, that was found by Nelson (1989), might be explained by a decreased ability of the pre-exposed animals in comparison to the naive fish, to respond to more severe acid conditions. Interestingly, in the experiments that were performed in soft

water, Nelson (1989) found a doubling of the variance in critical swimming speed under acid conditions, similar to our results in the present study.

We did not find a difference in oxygen uptake between our two experimental groups at rest. Under freshwater conditions at neutral pH the cost of ion regulation amounts to about 20% of the total metabolism (Rao 1968; Farmer and Beamish 1969; see Febry and Lutz 1987, for discussion). Unfortunately, there are no data on this percentage for fish in acid water. The results of the present study indicate that for juvenile carp the cost of ion regulation is not enhanced in water of pH 5.0. Although instant acid exposure of fish may induce passive ion losses, long-term exposure to low pH leads to a reduction of passive ion losses caused by a decreased ion permeability of the body surface (McWilliams 1980; Wendelaar Bonga et al. 1984). Flik et al. (1989) have shown that in tilapia, exposed for six months to acid water, not only the sodium efflux but also the active uptake of sodium was decreased. Such adaptive processes might reduce the metabolic costs of ion regulation, and thus the oxygen consumption.

An important result of this study is that there was no difference in oxygen consumption between the two experimental groups at swimming speeds in the range of 1.5 - 3.5 $BL \cdot s^{-1}$. This is in contrast to results of Waiwood and Beamish (1978), who found with rainbow trout, swimming at various speeds, that the oxygen consumption was increased at pH 6.0. At the start of the exercise period, when the fish were swimming at 1 $BL\ s^{-1}$, the pH 5.0 group had a higher oxygen uptake than the control group. We observed that these animals swam irregularly during this early phase of the experiment. Possibly the fish at pH 5.0 were more alarmed by the start of the experimental procedure than the control animals.

Juvenile carp can swim in acid water at 80% of their critical speed, without increasing their oxygen uptake to a higher extent than carp swimming at this speed in neutral water. In addition, for both groups of fish we found that the respiratory rate during rest prior to, and immediately after the

exercise period were similar, leading to the conclusion that swimming at this subcritical speed at pH 5.0 water does not cause an oxygen debt, which means that the anaerobic metabolism is not activated under these conditions. This result is a strong indication that, after 4 weeks exposure to pH 5.0, the ability of carp to perform subcritical exercise is not affected.

The plasma lactate concentrations after 15 minutes exhaustive exercise of the two groups were at a similar level, but were highly elevated in comparison to aerobic swimming cannulated carp (0.2 mmol l^{-1}; see chapter 4). So both groups use anaerobic metabolism to approximately the same extent to cover their energy need. The oxygen consumption during this period of strenuous exercise was lower at pH 5.0 than at pH 7.6, but we did not observe that the fish exercised less intensively under acid conditions. Ultsch, Ott and Heisler (1980) found that carp does not decrease its standard metabolic rate in the water pH range of 7.4 - 4.0. Our data from an earlier study on carp confirm this result (see chapter 5). So we do not believe that carp depressed their metabolic rate as a compensatory mechanism during our experiments at pH 5.0. This indicates that, at speeds above the critical velocity, long term pH 5.0 exposed carp, swim with higher efficiency than control fish. Measuring the tail stroke frequency and amplitude could be a first step to investigate a possible difference in swimming style between the two groups. From our observations of the swimming fish, it seemed that the animals in the acid group had a smaller stroke amplitude than the control fish, which might reduce swimming drag.

Fifteen minutes of strenuous exercise at pH 5.0 led to a 19% higher haematocrit value compared to animals swimming at the same speed in water of pH 7.6. In trout a significant increase of this parameter was observed after strenuous exercise in neutral water compared to resting fish (Turner et al. 1983). The rise in haematocrit after strenuous exercise is probably caused by splenic contraction, as was observed in various fish species (Yamamoto, 1988). Nilson and Grove (1974) showed that injection of catecholamines into the splenic artery contracted the spleen

of the cod, Gadus morhua. Considering that an increased haematocrit might be a result of enhanced catecholamine levels via adrenergic induced spleen contraction, the higher value in exercising carp at low pH might reflect that exercise is more stressful to the animal in acid than in neutral water, and this is in contrast with the conclusions on the above mentioned parameters measured in the present experiment. A second indication that acid water affects carp is the finding that the plasma chloride concentration at the end of the experiment was lower in the pH 5.0 group than in the controls. This points towards ionoregulatory imbalance. The mean plasma sodium concentration was also lower, but not significantly different from the controls. In our earlier studies with adult carp exposed to pH 4.0 (see chapters 3 and 4), we found that plasma sodium and chloride were not affected in resting animals, after 24 hours of steady state exercise, however, both ions were decreased. From our data it can not be deduced whether the ionoregulatory imbalance only took place during the 15 minutes exhaustive exercise or also before. Nevertheless, the higher haematocrit value and this ionoregulatory imbalance are the only indications in the present study that prolonged exposure to pH 5.0 has negative effects on carp. From the other data the conclusion may be drawn that water of pH 5.0 is rather well tolerated by carp. This is in line with the results of Ultsch and co-workers (Ultsch, Ott, and Heisler 1980 and 1981), who found that instant acidification to approximately pH 5 had little effect on oxygen consumption, acid/base status and ion balance in carp. In the present study we demonstrated that at this pH even strenuous exercise, as an additional stressor, does not have dramatic effects. We have concluded from our earlier studies on carp (see chapters 3 and 4) that the effects of acid water on fish have often been overestimated in the literature, probably because most laboratory studies apply instant acidification of the water, short-term exposure periodes, and handling. This conclusion is in line with the present result. However, although our experiments on juvenile and adult carp indicate that acid water is tolerated to a high extent, studies

on eggs and larval stages have shown that the early life stages of carp are very sensitive to water of pH 5.0 (Leuven et al. 1987, Oyen et al. 1990). Thus, natural carp populations are threatened at this pH by recruitment failure. Moreover, acidification of natural waters is often associated with an increase of the concentration of metals, such as aluminium. The aluminium hydroxides formed in water of around pH 5.0 are very toxic to fish (Wood and McDonald 1987; Witters 1990).

REFERENCES

Audet, C., and C.M. Wood. 1988. Do rainbow trout (Salmo gairdneri) acclimate to low pH? Canadian Journal of Fisheries and Aquatic Siences 45:1399-1405.

Audet, C., R.S. Munger, and C.M. Wood. 1988. Long-term sublethal acid exposure in rainbow trout (Salmo gairdneri) in soft water: effects on ion exchanges and blood chemistry. Canadian Journal of Fisheries and Aquatic Siences 45:1387-1398.

Beamish, F.W.H. 1978. Swimming capacity. Pages 101-187 in W.S. Hoar and D.J. Randall, eds. Fish physiology. Vol. VII. Academic Pres, New York.

Blazka, P., M. Volf, and M. Cepela. 1960. A new type of respirometer for the determination of the metabolism of fish in an active state. Physiologia Bohemooslova 9:553-560.

Butler, P.J., N. Day, and K. Namba. 1992. Interactive effects of seasonal temperature and low pH on resting oxygen uptake and swimming performance of adult brown trout Salmo trutta. Journal of experimental Biology 165:195-212.

Farmer, G.J., and F.W.H. Beamish. 1969. Oxygen consumption of Tilapia nilotica in relation to swimming speed and salinity. Journal of Fisheries Research Board of Canada 26:2807-2821.

Febry, R., and P. Lutz. 1987. Energy partitioning in fish: the activity related cost of osmoregulation in a euryhaline

cichlid. Journal of experimental Biology 128:63-85.

Flik, G., J.A. Van der Velden, C.M. Seegers, Z. Kolar, and S.E Wendelaar Bonga. 1989. Prolactin cell activity and sodium fluxes in tilapia (Oreochromis mossambicus) after long-term acclimation to acid water. General and Comperative Endocrinology 75:39-45.

Graham, M.S., and C.M. Wood. 1981. Toxicity of environmental acid to the rainbow trout: interactions of water hardness, acid type, and exercise. Canadian Journal of Zoology 59:1518-1526.

Graham, M.S., C.M. Wood, and J.D. Turner. 1982. The physiological response of the rainbow trout to strenuous exercise: interactions of water hardness and environmental acidity. Canadian Journal of Zoology 60:3153-3164.

Leuven, R.S.E.W., S.E. Wendelaar Bonga, F.G.F. Oyen, and W. Hagemeijer. 1987. Effects of acid stress on the distribution and reproductive success of freshwater fish in Dutch soft waters. Annales de la Societe Royale Zoologique de Belgique 117:231-242.

McWilliams, P.G. 1980. Acclimation to an acid medium in the brown trout, Salmo trutta. Journal of Experimental Biology 88:269-280.

Nelson, J.A. 1989. Critical swimming speeds of yellow perch Perca flavescens: comparison of populations from a naturally acidic lake and a circumneutral lake in acid and neutral water. Journal of experimental Biology 145:239-254.

Nelson, J.A. 1990. Muscle metabolite response to exercise and recovery in yellow perch (Perca flavescens): comparison of populations from naturally acidic and neutral waters. Physiological Zoology 63:886-908.

Nelson, J.A., and G.S. Mitchell. 1992. Blood chemistry response to acid exposure in yellow perch (Perca flavescens): comparison of populations from naturally acidic and neutral environments. Physiological Zoology 65:493-514.

Nilsson, S., and D.J. Grove. 1974. Adrenergic and cholinergic innervation of the spleen of the cod: Gadus morhua. European Journal of Pharmacology 28:135-143.

Oyen, F.G.F., L.E.C.M. Camps, and S.E. Wendelaar Bonga. 1990. Effect of acid stress on the embryonic development of the common carp (Cyprinus carpio). Aquatic Toxicology, 19:1-12.

Rao, G.M.M. 1968. Oxygen consumption of rainbow trout (Salmo gairdneri) in relation to activity and salinity. Canadian Journal of Zoology 46:781-786.

Smit, H., J.M. Amelink-Koutstaal, J. Vijverberg, and J.C. Von Vaupel-Klein. 1971. Oxygen consumption and efficiency of swimming goldfish. Comparative Biochemistry and Physiology 39A:1-28.

Turner, J.D., C.M. Wood, and D. Clark. 1983. Lactate and proton dynamics in the rainbow trout (Salmo gairdneri). Journal of Experimental Biology 104:247-268.

Ultsch, G.R., M.E. Ott, and N. Heisler. 1980. Standard metabolic rate, critical oxygen tension, and aerobic scope for spontaneous activity of trout (Salmo gairdneri) and carp (Cyprinus carpio) in acidified water. Comparative Biochemistry and Physiology 67A:329-335.

Ultsch, G.R., M.E. Ott, and N. Heisler. (1981). Acid-base and electrolyte status in carp (Cyprinus carpio) exposed to low environmental pH. Journal of Experimental Biology 93:65-80.

Waiwood, K.G., and F.W.H. Beamish. 1978. Effects of copper, pH and hardness on the critical swimming performance of rainbow trout (Salmo gairdneri Richardson). Water Research 12:611-619.

Wendelaar Bonga, S.E., J.C.A. Van der Mey, and G. Flik. 1984. Prolactin and acid stress in the teleost Oreochromis (formerly Sarotherodon) mossambicus. General and Comparative Endocrinology 55:323-332.

Wendelaar Bonga, S.E., G. Flik, and P.H.M. Balm. 1987. Physiological adaptation to acid stress in fish. Annales de la Societe Royale Zoologique de Belgique 117:243-254.

Witters, H. 1990. Physiological study on the waterbug, Corixa punctata and the trout, Oncorhynchus mykiss: effects of aluminium in acid water. Ph.D. thesis, University of Antwerp, Belgium.

Wood, C.M. 1989. The physiological problems of fish in acid waters. Pages 125-152 in R. Morris, D.J.A. Brown, E.W. Taylor, and J.A. Brown, eds. Acid Toxicity and Aquatic animals. Cambridge University Press, Cambridge.

Wood, C.M., and D.G. McDonald. 1987. The physiology of acid/aluminium stress in trout. Annales de la Societe Royale Zoologique de Belgique 117:399-410.

Yamamoto, K. 1988. Contraction of spleen in exercised freshwater teleost. Comparative Biochemistry and Physiology 89a:65-66.

Ye, X., and D.J. Randall. 1991. The effect of water pH on swim ming performance in rainbow trout (Salmo gairdneri, Richardson). Fish Physiology and Biochemistry 9:15-21.

Ye, X., D.J. Randall, and X. He. 1991. The effect of acid water on oxygen consumption, circulating catecholamines and blood ionic and acid-base status in rainbow trout (Salmo gairdneri, Richardson). Fish Physiology and Biochemistry 9:23-30.

CHAPTER 7:

SUMMARY AND GENERAL DISCUSSION

This thesis deals with the physiological response to acid exposure of two teleost freshwater fish: tilapia (Oreochromis mossambicus) and common carp (Cyprinus carpio).

We first studied the effect of gradual water acidification on resting fish (chapters 2 & 3). Gradual acidification was applied for two reasons: first, in order to prevent epithelial damage that occurs after instantaneous acidification; second, because studies applying gradual acidification can be expected to give better insight in the tolerance of acid water of the species concerned and because in nature acidification is also gradual rather than immediate. As in most studies on the effects of acid exposure on fish, we examined the electrolyte, and the acid/base balance. A new aspect of our study was the investigation of the energy status and the acid/base balance under these conditions by *in vivo* ^{31}P-NMR spectroscopy. In tilapia, gradual water acidification to pH 4.0 caused a transient drop of the pH of the blood plasma (0.24 units) and the gill epithelium (0.19 units) but the pH of both compartments slowly recovered during the 10-h period of exposure to water of pH 4.0. In contrast, the pHi of muscle tissue was only slightly affected. Alkalinization of the water caused a remarkable transient decline of the plasma pH. Protons that had entered the fish during the acid exposure period were possibly temporarily stored in a buffer, such as calcium carbonate (in scales and bone). The decline in plasma pH could then be explained by release of stored acid equivalents into the blood. The high-energy phosphate stores in the tissues remained at control level during the experiment.

In carp, fitted with arterial catheters, acid exposure to pH 4.0 caused remarkable little disturbance of the measured plasma parameters (e.g.: pH_e and ion concentrations), which is

in contrast to the literature data, including those on carp. Since we applied gradual water acidification, in contrast to instant drops in water pH employed in previous studies, we conclude that the rate of water acidification rather than the pH level itself determines the early effects of acid exposure. Another new aspect of our study is that we monitored the plasma catecholamine levels over 48 hours. They remained at control level throughout the experiment, which is an indication that our experimental procedure was not very stressful for the animals. The plasma cortisol level, however, showed a transient increase, which is typical for the primary response of fish to stressors, indicating that the acid exposure still was experienced as a stressor in our experiment.

In the second part of this study on acid exposure (chapters 4, 5 & 6), we observed our experimental fish while performing various forms of exercise: spontaneous, steady state, or strenuous exercise. This type of experiment can be considered more realistic from an environmental point of view than experiments with resting fish; in the wild fish are not constantly at rest but swim actively in a variety of behaviours such as feeding, avoiding predators, and migrating. In contrast to carp at rest, after 24 hours of acid exposure (pH 4.0), steady state exercising carp showed a slight hyperglycemia and a constant decline of plasma pH and of the plasma sodium and chloride concentration, indicating ionoregulatory failure (chapter 4). We conclude that there is a synergistic effect of gradual water acidification and steady state exercise on carp. Similar to our results with carp at rest, plasma catecholamines of swimming carp remained at control level, while plasma cortisol showed a transient increase.

Continuous oxygen consumption measurements were carried out on carp and tilapia, which were allowed to perform routine activity (chapter 5). The main conclusion of this study is that the tilapia decreases its standard metabolic rate, its average oxygen consumption, and its maximum metabolic rate in water of pH 4.0, whereas the carp does not. A comparison of our results with data from the literature revealed that the effect of acid

water on the oxygen uptake of carp was not influenced by the acidification procedure (i.e. gradual versus instantaneous). Interestingly, in contrast to tilapia, carp sometimes reacted to water acidification with an extreme peak in oxygen consumption. Tilapia became completely motionless in water of low pH. Our explanation is that the two species have different strategies to react to acid exposure: Whereas the carp reacts with an attempt to escape, the tilapia avoids the additional stress of exercise (see above). The ability of carp to perform exercise in acid water was tested in the experiment that was described in chapter 6. Groups of juvenile carp were held for at least four weeks in water of pH 5.0 or 7.6. We did not find a significant difference in critical swimming speed between the two groups. Nor was there an effect of acid exposure on oxygen consumption at most swimming speeds, so we conclude that the ability of carp to perform exercise is not affected by four weeks of acid exposure. The O_2 uptake measurements and the plasma lactate levels indicated that carp swim at a higher efficiency in acid than in neutral water. The only indications found in this study that long-term acid exposure had negative effects on carp were a higher haematocrit and a lower plasma chloride concentration in the pH 5.0 group after strenuous exercise.

From the results described in this thesis, we conclude that the physiological effects of acid exposure of carp have been overestimated in the literature. Application of short-term exposure periods or additional stress, such as handling or an instant acidification procedure, or the (unnoticed) presence of aluminium in the water, probably caused this overestimation. This might also apply to other fish species, so the literature has to be critically viewed in this respect. A strong indication that our conclusion based on the results with carp might also be valid for other species comes from a recent study with rainbow trout (Balm & Pottinger, 1993; Can. J. Fish. Aq. Sci., accepted). The authors reported only minor disturbances in the measured blood parameters after gradual water acidification. In conclusion, in future investigations on the effect of acid water *per se* on the physiology of fish, a gradual rather than an acute

water acidification procedure should be applied.

DE FYSIOLOGISCHE REACTIE VAN VISSEN OP WATERVERZURING

Dit proefschrift behandelt de fysiologische effecten van waterverzuring op twee soorten zoetwatervis: de natalbaars, ook wel tilapia genoemd, (Oreochromis mossambicus) en de gewone karper (Cyprinus carpio).

Als eerste hebben we naar de effecten van geleidelijke waterverzuring op vissen in rust gekeken. Geleidelijke verzuring werd om twee redenen toegepast. Ten eerste, om beschadiging van het kieuwepitheel te voorkomen die plaatsvindt na acute verzuring. Ten tweede, omdat van studies die deze vorm van verzuring toepassen te verwachten valt dat ze een beter inzicht geven in de zuurbestendigheid van de betreffende vissoort, aangezien in de natuur waterverzuring ook geleidelijk verloopt, niet acuut. Zoals in de meeste studies naar de effecten van blootstelling aan verzuurd water op vissen, hebben we naar de ionenhuishouding en de zuur/base-balans gekeken. Nieuw aan onze studie is dat we voor onderzoek aan de zuur/base-balans en de energiehuishouding gebruik hebben gemaakt van in vivo ^{31}P-NMR-spectroscopie (hoofdstuk 2). Geleidelijke waterverzuring tot pH 4,0 veroorzaakte in tilapia een kortstondige daling van de pH van het bloedplasma (0,24 pH-eenheden) en van het kieuwepitheel (0,19 pH-eenheden), die zich echter beide nog tijdens de tien uur zuurblootstelling herstelden. Dit in tegenstelling tot de intracellulaire pH van het spierweefsel, die hoegenaamd geen daling vertoonde. Verhoging van de water-pH tot 7,6 veroorzaakte een opmerkelijke kortstondige daling van de plasma-pH. Mogelijkerwijs waren de protonen, die de vis tijdens de periode in zuur water opgenomen had, tijdelijk opgeslagen in een inwendige buffer. In dat geval, kan de daling van de plasma-pH verklaard worden door de uitstoot van H^+-ionen in het bloed. Tijdens dit experiment bleven de in de weefsels voorkomende energierijke fosfaatverbindingen op controlenivo.

In gecanuleerde karpers veroorzaakte blootstelling aan

verzuurd water opmerkelijk weinig verstoringen in de gemeten plasmaparameters (b.v.: pH_e en ionen; hoofdstuk 3), dit in tegenstelling tot wat er beschreven is in de literatuur, met inbegrip van gegevens over de karper. Aangezien wij de water-pH geleidelijke verlaagd hebben, terwijl in de literatuurstudies een acute vorm van verzuring is toegepast, concluderen wij dat de snelheid waarmee de pH van het water daalt, in hoge mate bepalend is voor de vroegtijdige effecten van zuurblootstelling. Een nieuw aspect van deze studie is dat we gedurende het gehele experiment het verloop van de plasmacatecholamines gevolgd hebben. Zowel adrenaline als noradrenaline bleven op controlenivo, hetgeen een indicatie is dat onze experimentele procedure niet erg stressvol was voor de dieren. Dat de waterverzuring toch als een stressor op de beesten gewerkt heeft, blijkt uit de plasmacortisolmetingen. Deze vertoonde een tijdelijke verhoging, hetgeen typerend is voor de primaire reactie van vissen op stress.

Het tweede gedeelte van dit proefschrift (hoofdstuk 4, 5 & 6) behandelt de fysiologische effecten van verzuurd water op vissen, tijdens activiteit. Verscheidene vormen van zwemmen zijn gebruikt: routine-activiteit, steady-state zwemmen en uitputtend zwemmen. Dit type experimenten komt meer met de natuurlijke situatie overeen, waar de vissen ook niet continu in rust verkeren, maar in het kader van verscheidene gedragspatronen, zoals voedsel zoeken, het vermijden van roofdieren of tijdens de vistrek, actief zijn. In tegenstelling tot de situatie bij karpers in rust, vertoonden de steady-state zwemmende karpers wel duidelijke verstoringen in de gemeten bloedparameters (hoofdstuk 4). 24 Uur bij pH 4,0 veroorzaakte een milde vorm van hyperglycemie, een geleidelijke daling van plasma-pH en van de plasmaconcentraties van natrium en chloride. Het laatste wijst op een verstoring van de ionenregulatie. Hieruit concluderen wij dat geleidelijke waterverzuring en steady-state zwemmen een synergystisch effect hebben op de gewone karper. Overeenkomstig met onze resultaten met karpers in rust, vonden we met zwemmende karpers, onveranderde plasmaconcentraties van adrenaline en noradrenaline en een voorbijgaande verhoging van plasmacortisol.

Tijdens routine-activiteit, werden continue zuurstof-verbruiksmetingen uitgevoerd met de natalbaars en de gewone karper (hoofdstuk 5). De belangrijkste conclusie van deze studie is dat tilapia, in tegenstelling tot karper, in zuur water (pH 4,0) zijn standaardmetabolisme, zijn gemiddelde zuurstof-consumptie en zijn maximale metabolisme verlaagt. Uit een vergelijking van onze gegevens met informatie uit de literatuur bleek, dat het effect van waterverzuring op de zuurstofopname, onafhankelijk is van de gevolgde verzuringsprocedure (geleidelijk dan wel acuut). Een interessant resultaat van deze studie was ook dat de karper op waterverzuring reageerde met een piek in de zuurstofopname. Dit in tegenstelling tot tilapia die zich volkomen rustig hield in zuur water. Onze verklaring hiervoor is dat de twee vissoorten verschillende strategieën ontwikkeld hebben om op waterverzuring te reageren. Terwijl de karper probeert om aan het zure water te ontkomen, houdt de tilapia zich rustig om de bijkomende stress van activiteit te voorkomen (zie hiervoor). Het zwemvermogen van karper in zuur water werd onderzocht in de studie die in hoofdstuk 6 beschreven staat. Groepen onvolwassen karpers werden tenminste vier weken in water met een pH van 5,0 of 7,6 gehouden. Er werd geen significant verschil gevonden in kritische zwemsnelheid tussen de twee groepen. Aangezien er ook geen effect gevonden werd op de zuurstofopname bij de meeste zwemsnelheden, concluderen wij dat het zwemvermogen van de karper op nivo blijft na vier weken blootstelling aan verzuurd water. De zuurstofverbruiksmetingen en het plasmalactaatgehalte indiceerden dat de karper, bij zwemsnelheden boven de kritieke snelheid, efficienter zwemt in zuur, dan in neutraal water. De enige indicaties, die in deze studie gevonden zijn, die er op duiden dat langdurige blootstelling aan verzuurd water negatieve effecten op de karper heeft, zijn een hogere haematocritwaarde en een lagere concentratie van de plasmachloride, gemeten in de zuur-blootgestelde vissen na uitputtende zwemarbeid.

Op grond van de resultaten die in dit proefschrift beschreven zijn, concluderen wij dat in de literatuur de fysiologisch effecten van blootstelling aan verzuurd water op

vissen overschat zijn. De volgende factoren hebben tot deze overschatting geleid: het toepassen van kortdurende zuurblootstelling, dan wel bijkomende stressoren zoals handling, de aanwezigheid van aluminium in het water, of een acute verzuringsprocedure.

Zoals voor alle dingen in het leven geldt, geldt ook hier dat je alléén niets kunt voortbrengen. Daarom wil ik alle mensen, die in enige vorm aan het tot stand komen van dit proefschrift bijgedragen hebben, bedanken voor hun hulp: mijn promotor, prof. dr. S. Wendelaar Bonga, en mijn co-promotor, dr. G. van den Thillart, voor hun goede raad en vooral voor hun geduld, Iris voor haar steun en stimulatie en Niklas voor zijn significante positieve werking op mijn stemming. Voor de interesse die ze door de jaren getoond hebben, wil ik mijn familie en vrienden hartelijk danken. De mensen van de afdeling Dierfysiologie in Leiden en van de gelijknamige afdeling in Nijmegen dank ik voor de prettige samenwerking. Voor hun gewaardeerde hulp bij de correctie van het proefschrift bedank ik tenslotte nog Wim van Dijk en Marjorie Hardison.

Peter van Dijk werd op 8 juni 1958 in Schiedam geboren. Tussen 1977 en 1982 werd met succes de lerarenopleiding ZWN, Delft, gevolgd voor de vakken biologie en natuurkunde. In 1981 werd een aanvang gemaakt met de studie biologie aan de Rijksuniversiteit van Leiden. Het doctoraalexamen met het hoofdvak dierfysiologie (dr. G. van den Thillart), het bijvak ethologie (prof. dr. P. Sevenster en dr. M. 't Hart) en de onderwijsbevoegdheid biologie, werd in mei 1986 behaald. Tijdens de doctoraalfase werd voor het hoofdvak een studiebezoek gebracht (november 1984 - juli 1985) aan het laboratorium van prof. dr. C. Wood, McMaster University, Hamilton, Canada. Onder zijn leiding werd ook een maand onderzoek verricht in de Verenigde Staten (Friday Harbor Laboratories, University of Washington, WA). Tevens werd, onder leiding van dr. G. van den Thillart, een studiebezoek gebracht (september - oktober 1985) aan het laboratorium van prof. dr. N. Heisler, Max-Planck Institut für Experimentelle Medizin, Göttingen, Duitsland. Van augustus 1986 tot augustus 1989 was hij als wetenschappelijk onderzoeksmedewerker in dienst van BION/NWO. In die functie werd, bij de afdeling Dierfysiologie van de Rijksuniversiteit Leiden en bij de afdeling Dierfysiologie van de Katholieke Universiteit Nijmegen, het in dit proefschrift beschreven onderzoek verricht. Momenteel is hij als leraar verbonden aan de International School of Düsseldorf, Duitsland.

STELLINGEN

1 Het gebruiken van gegevens uit de literatuur betreffende de gevolgen van verzuring op de fysiologie van vissen dient kritisch te gebeuren Er zal bekeken moeten worden in hoeverre bijkomende stressoren - zoals handling, de aanwezigheid van metalen zoals aluminium in het water, of een acute verzuringsprocedure - tot een overschatting van de negatieve effecten geleid kunnen hebben

Dit proefschrift

2 De door Ultsch, Ott en Heisler gevonden verstoringen in de ionenhuishouding en de zuur/base-balans in karpers in verzuurd water zijn mede te wijten aan de door hun gebruikte acute verzuringsprocedure

G R Ultsch et al. (1981) J exp Biol 93, 65-80

3 Geleidelijke waterverzuring en steady-state zwemmen hebben een synergistisch effect op de fysiologie van de gewone karper

Dit proefschrift

4 Tilapia verlaagt, in tegenstelling tot de gewone karper, zijn standaardmetabolisme in verzuurd water

Dit proefschrift

5 De functie van groeihormoon bij vissen is onduidelijk

J P Sumpter et al. (1991) Gen Comp Endocrinol 83, 94-102

6 Ten onrechte wordt zonne-energie als een niet-rendabele energiebron afgedaan Wanneer alle kosten in aanmerking genomen worden dan kan deze vorm van electriciteit-opwekken goedkoper zijn dan kernenergie

7 Voor de vooruitgang van de wetenschap is het noodzakelijk dat er spoedig een zo volledig mogelijk, eenvoudig toegankelijk en gecomputeriseerd literatuursysteem ingevoerd wordt.

8 Gezien de grote maatschappelijke schade die alcoholische dranken en tabakswaren veroorzaken, is het onbegrijpelijk dat er nog steeds reclame voor deze verslavende middelen gemaakt mag worden

9 Wanneer we aannemen dat een geweten toetsbaar is, dan is het voor een land zinvoller om het geweten van kandidaten voor leidinggevende functies binnen het leger te toetsen, dan dat van gewetensbezwaarde dienstplichtigen

10 Gelukkig is er één milieuprobleem waarvan de oplossing in zicht is Met het opraken van de wereldolievoorraad wordt het probleem van de lekkende, lozende en/of zinkende olietankers vanzelf opgelost

Peter van Dijk Dusseldorf, 5 mei 1993